AN ABSTRACT OF THE THESIS OF

Somkiat Kanchanakhan for the degree of Master of Science in Microbiology presented on November 30, 1990.

Title: Hematology and Histopathology of Coho Salmon (Oncorhynchus kisutch) Infected with Flexibacter psychrophilus

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Abstract approved: _____
Dr. John L. Fryer

Flexibacter psychrophilus grew best in tryptone yeast infusion and tryptone yeast extract broth plus salts at 18±2°C with shaking at 130 rpm. The medium lethal dose of the bacterium within 15 days was 2.3 x 10^7 cells/ml for coho salmon (Oncorhynchus kisutch) averaging 21.8 gm. Coho salmon injected intramuscularly with 9.4 x 10^5 and 2.5 x 10^7 bacterial cells/ml showed severe disease at day 7 and days 7 to 10, respectively. In the early stages of the disease, percent and absolute count of monocytes significantly decreased from 6.0 to 1.0% and 580 to 80 cells/mm^3, respectively. In the severe stages of the disease, hematologic parameters had significantly different means (control values are in the parentheses); hematocrit was 31.0 (39.9%); red blood cell 1.06 x 10^6 (1.51 x 10^6 cells/ml); and white blood cell 2.6 x 10^3 (9.7 x 10^3 cells/ml). The total plasma protein was 1.25 (4.0 gm/dl); hemoglobin 8.23 (9.96 gm/dl); plasma glucose, mean corpuscular volume, and mean

corpuscular hemoglobin had the highest values at 139.3 mg/dl, 368 μm^3/cell, and 83 pg/cell, respectively. Mean corpuscular hemoglobin concentration decreased to 23.4% and the percent of thrombocytes increased to 51.2 (37.0%). In the early recovery stages, the absolute count of lymphocytes and neutrophils increased from 4.5 x 10^3 to 9.0 x 10^3 and 0.4 x 10^3 to 1.3 x 10^3, respectively. The percentage of lymphocytes increased from 42 to 70%.

Flexibacter psychrophilus-injected coho salmon showed swelling and open lesions. The bacterial cells spread between myofibrils at the site of injection and caused necrosis, blood capillary invasion, inflammation, hemorrhage, atrophy, and edema. The bacterium caused histologic changes of the following organs: the gills with hypertrophy, edema, and hemorrhage; the liver with vacuolar degeneration, pycnotic nuclei, congestion, and hemorrhage; the spleen with ellipsoidal necrosis, increasing hemosiderin, and vacuolar degeneration; the kidney with decreasing hemopoietic tissue, increasing hemosiderin, necrosis of renal tubules, and congestion; the pancreas with necrosis of acinar and islets cells; the heart showed some evidence of focal vacuolation in cardiac muscle, increasing and swelling of endocardial macrophages; and the intestines with dilation of cecal villi.

These studies indicate F. psychrophilus is a cause of hypoplastic anemia. The bacterial cells could not be found in the surviving fish at 19 days post injection.

Hematology and Histopathology of Coho Salmon (*Oncorhynchus kisutch*) Infected with *Flexibacter psychrophilus*

by

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A THESIS

submitted to

Oregon State University

in partial fulfillment of
the reguirements for the
degree of

Master of Science

Completed November 30, 1990

Commencement June 1991

APPROVED:

Redacted for Privacy

Professor and Chairman of the Department of Microbiology in charge of major

Redacted for Privacy

Dean of the Graduate School

Date thesis is presented November 30, 1990

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ACKNOWLEDGEMENTS

I would like to express my appreciation to Prof. John Fryer for his encouragement and patience. He not only be available when I need but also solve problems that I usually have. To Dr. Kamonporn Tonguthai and personal at National Inland Fisheries Institute, Bangkok, Dr. Brian Davy, Dr. Richard Arthur, Dr. Andrew McNaughton, Kim Dalay, Mary Beaussart, Tan Say Yin, and personal at International Development Research Centre (IDRC), head office at Ottawa and regional office at Singapore, who helped me throughout this program.

A special thanks to Dr. John Rohovec, Dr. Robert Olson, and Dr. Richard Holt for the valuable assistance and thesis correction.

To Jiraporn Kasornchandra for review some classes before the exam, to Harriet Lorz for histologic assistance to Jerri Bartholomew for photo-taking technique.

To Thirapon Thiragirayuta for major typing of thesis, to Thirapon and Chutikarn Kitprasert for dinner meals during the writing period of thesis.

Finally, to my parents Sewhong and Keaheing Kanchanakhan and to my sisters and brothers for their tremendous encouragement.

This study program was supported by IDRC under project Fish Disease (Thailand) and the Department of Microbiology, Oregon State University.

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HEMATOLOGY AND HISTOPATHOLOGY OF COHO SALMON (ONCORHYNCHUS KISUTCH) INFECTED WITH FLEXIBACTER PSYCHROPHILUS

INTRODUCTION

Flexibacter psychrophilus is a cytophagal fish pathogen which causes bacterial cold-water disease (BCWD). The disease was first reported by Davis (1946). The etiological agent was successfully isolated and cultured from infected fingerling coho salmon in spring 1948 and named as a new bacterial species, "Cytophaga psychrophila," by Borg (1960). For the last two decades, bacterial taxonomists have worked to reclassify "C. psychrophila" and the genus Cytophaga. Recently, this bacterium was renamed Flexibacter psychrophilus by Bernardet and Grimont (1989) and included in the approved lists of bacterial names in the International Journal of Systematic Bacteriology since 1989.

Flexibacter psychrophilus is a slender, rod-shaped, gram-negative bacterium that causes serious loss of hatchery-reared salmonids, especially in the northwestern USA. The infected fish usually show a lesion at the caudal peduncle with exposed muscle and/or vertebral column and hemorrhage of internal organs. Many strains of F. psychrophilus that are closely related to the original strain of Borg (1960) have been isolated from salmonids in North America, Europe, and East Asia. This study used strain SH3-81, isolated from infected coho salmon at the Sandy Hatchery, Oregon,

during an epidemic of BCWD in 1981. The virulence of this strain was determined by inoculation of fish and is expressed as the LD_{50}.

There is no report of hematologic changes in infected coho salmon with BCWD. This study examined the changes in hematology and histopathology during the development of the disease. Vaccination against BCWD was successful on a laboratory scale (Holt, 1987), so an investigation of different culture media was included in this study to provide information for mass production of bacteria.

The objectives of this study were to: (1) select the best growth media, incubation temperature, and shaking rate for F. psychrophilus; (2) test the virulence of the bacterium by using the LD_{50} method; (3) examine the hematologic and histopathologic changes during the development of the disease.

LITERATURE REVIEW

Bacterial cold-water disease is a serious infection of salmonid fish. The first description of BCWD was reported by Davis (1946) in fingerling rainbow trout (Salmo gairdneri now Oncorhynchus mykiss) at Leetown, West Virginia, under the name of "peduncle disease". The disease was first recognized during late spring and early summer 1941 and reoccurred during the spring of 1945. Infected rainbow trout had lesions exposing the muscle and vertebral column beneath the caudal peduncle (Davis, 1946). Davis (1946) reported that this unique disease had not been reported in salmonids from other locations (hatcheries). In the lesions he found numerous, non-motile, slender, rod-shaped bacterial cells, 3 to 5 μm in length. Unfortunately, he was unable to culture the microorganism on bacteriological media.

The etiological agent of BCWD was successfully isolated from the organs and lesions of diseased fingerling coho salmon (Oncorhychus kisutch) at the Minter Creek Hatchery, Washington Department of Fisheries in April 1948 (Borg 1960). He proposed that this bacterium be placed in the genus Cytophaga because its morphologic appearance was similar to Cytophaga johnsonae, described by Stainer (1947). Borg (1960) also reported the etiological agent of BCWD as a new species because it had a thin spreading colony and certain biochemical characteristics distinct from known Cytophaga species. The species was named "psychrophila" because it caused disease at low water temperatures (6 to 10°C). "Cytophaga psychrophila," was suggested as a new

species in the genus Cytophaga (Borg, 1960). The disease is very similar or identical to peduncle disease of fingerling rainbow trout (Holt, 1987; Pacha and Ordal, 1970). At water temperatures between 6 and 10°C, heavy losses of coho salmon infected with BCWD have been reported. Borg (1960) states that of the one million fish in all ponds, losses exceeded 30% within the first two months and in one pond was more than 53%.

The characteristics of "C. psychrophila" have been reported by Borg (1960) and Pacha (1968). Morphologically, the organism is a gram-negative, flexible rod that is 0.75 µm in width and 1.5 to 7.5 µm in length and is motile by means of gliding. It forms bright yellow to golden, lipid soluble pigment and colonies are 2 to 3 mm in diameter with a thin spreading margin on Cytophaga agar. It does not form fruiting bodies or microcysts but needs oxygen for growth. "Cytophaga psychrophila" has optimum growth at about 15°C (Holt, 1987) but reproduces within a temperature range of 4 to 23°C (Holt, 1972; Pacha, 1968). Because of the ability to grow at low temperatures, this organism is classified as a psychrophilic bacteria. Morita (1975) stated that this bacterium was the first well characterized psychrophilic bacterium studied before 1963.

The physiological characteristics of "C. psychrophila" have been reported by a number of researchers, Borg (1960), Pacha and Ordal (1970), Otis (1984), and Holt (1987). The bacterium does not degrade simple or complex carbohydrates but its proteolytic activity can breakdown gelatin, casein, albumin, and tyrosine. It produces catalase but does not produce hydrogen sulfide, acetylmethylcarbinol, or indole. It does not utilize citrate nor reduce

nitrate. Cytochrome oxidase is produced from the fresh culture (Bernardet and Kerouault, 1989). It requires a very simple nutritional medium; growth can be observed in 0.5% tryptone (Borg,1960) or in a vitamin-free casein hydrolysate medium (Pacha and Ordal, 1970; and Otis, 1984). Some strains of the bacteria can grow in 1% casamino acids and 0.01% sodium lauryl sulfate (Holt, 1987). Otis (1984) and Holt (1987) reported that most strains of "C. psychrophila" could digest the dead cells of Escherichia coli and Aeromonas hydrophila and could degrade chondroitin sulfate, collagen, fibrinogen, and fish muscle extract.

In the late 1960's, the classification and taxonomy of cytophagal bacteria was intensively studied. The bacteria were classified in the genus Cytophaga, because they could attack many polysaccharides, such as cellulose, chitin, agar, and alginate, which separated Cytophaga from Flexibacter and Microscilla (Lewin, 1969). The flexible cell walls and the absence of flagella and endospores in Cytophaga were useful in separating it from eubacteria (Lewin and Lounsbery, 1969). Mitchell et al. (1969) studied the taxonomy of Cytophaga sp. and reported that they had difficulty defining the characteristics of the genus Cytophaga because of all the bacteria named under this genus and because of the broad overlap with Flexibacter and other genera. They defined the genus Cytophaga and suggested that the bacteria that lacked these characteristics should be placed in a new or established genera. Remarkably, part of their definition included the ability to attack polysaccharides. The phylogenic position of "Cytophaga psychrophila" became uncertain, because the bacterium failed to attack agar and cellulose (Borg,

1960). Otis (1984) could not detect the degradation of chitin and cellulose by five strains of "C. psychrophila." Holt (1987) noted similar results with agar, cellulose, carboxymethyl cellulose, and chitin when tested against 28 strains of the bacterium. Similar results were also reported by Bernardet and Kerouault (1989) with two strains from the U.S. and five local strains from France. This bacterium did not appear in the eighth edition of Bergey's Manual of Determinative Bacteriology. Leadbetter (1974) classified those cytophagal bacteria which were unable to attack agar, alginate cellulose, and chitin as the genera Flexibacter, while bacteria in the genus Cytophaga could breakdown certain of these.

In phylogeny, "C. psychrophila" seemed to have been in an uncertain position between the genera Cytophaga and Flexibacter. Christensen (1977) noted that the genus Flexibacter varied in its ability to degrade certain polysaccharides. She suggested that the potential for polysaccharide degradation should be determined in the genera Cytophaga and Flexibacter before "C. psychrophila" and "F. columnaris" were renamed. Another means of classification, using G+C content and a menaquinone system (usually found in the gram-positive bacteria), was reported by Oyaizu and Komagata (1981). They suggested that the cytophagal bacteria, which have menaquinone (MK-6 or MK-7) and a low G+C content, should be classified in the genus Cytophaga. The most recent effort at classification was reported by Bernardet and Grimont (1989). They used "DNA relatedness" together with the G+C content of the DNA, morphological, physiological, and biochemical tests to classify the various strains of "F. columnaris," "C. psychrophila," and Flexibacter

maritinus. They reported that "C. psychrophila" strain SH3-81 and the five French strains formed a tight genomic species (>90% relatedness) with the type strain NCMB 1947 described by Borg (1960). Their results, however, did not clarify the Cytophaga-Flexibacter-Flavobacterium phylogenetic branch. Thus, they suggest, the species "psychrophila" and "columnaris" be classified in the genus Flexibacter as Flexibacter psychrophilus and Flexibacter columnaris, until more work is done. Since 1989, both bacteria have been included in the approved lists of bacterial names in the International Journal of Systematic Bacteriology. Bernardet and Grimont (1989) refined the description of F. psychrophilus through their experiments and the experiments of Borg (1960), Pacha (1968), and Holt (1972 and 1987) as:

> "Cells are nonsporulating, nonmotile, gram-negative rods that are 1 to 5 μm long by 0.3 to 0.5 μm wide in 48-h liquid cultures; a few longer cells (8 to 12 μm) may appear. Some cells have an enlarged end. In hanging-drop preparations, the gliding movement is frequently slow and weak and is noticed after prolonged observation. Some strains exhibit faster gliding. Good growth occurs in Anacker-Ordal broth (2) supplemented with 0 or 0.5% NaCl and at 10 to 20°C. The optimum temperature is 15 to 18°C, and scant, slow growth occurs at 6 and 22 to 25°C. No growth occurs in Tryticase soy broth. Growth on Anacker-Ordal agar is enhanced by an enriched formula (0.5% tryptone instead of 0.05% tryptone). Most colonies are smooth, glossy, and circular with regular edges, but most strains can also produce colonies with narrow and uneven spreading margins. The colonies are bright yellow and do not adhere to the agar. Nondiffusible flexirubin type pigments are present. Colonies do not absorb Congo red. Strictly aerobic. Catalase and cytochrome oxidase are produced. Nitrate is not reduced, and hydrogen sulfide is not produced. *o*-Nitrophenyl-β-D-galactopyranoside is not hydrolyzed. Cellulose, carboxymethyl cellulose, chitin, starch, esculin, and agar are not hydrolyzed. No acid is produced from carbohydrates in ammonium salt-sugar medium (API 50CH galleries). Gelatin, casein (skim milk agar), and tyrosine are hydrolyzed. No brown color is produced on tyrosine agar. Lysine, arginine, and ornithine are not decarboxylated. Tributyrin, lecithin (egg yolk), Tween 20, and Tween 80 are hydrolyzed. Weak, slow DNA hydrolysis. No

> inhibition zone is formed around the following types of disks (the concentration in each type of disk is indicated in parentheses): gentamicin (15 μg), neomycin (30 μg), polymysin B (30 μg), and trimethoprim (5 μg). Susceptible (inhibition zone, more than 9 mm) to vibriostatic compound O/129 (500 μg), ampicillin (10 μg), cephalothin (30 μg), streptomycin (10 IU), tetracycline (30 IU), chloramphenicol (30 μg), erythromycin (15 IU), novobiocin (30 IU), and furans (300 μg)..."

This description also included the hydrolysis of substrates by using commercially available enzyme detection system, API ZYM. This bacterium hydrolyzed six of 28 subtrates. The G+C content is 32.5 to 34.0 mol%. The type strain of this bacterium is NCMB 1947, part of the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland (Bernardet and Grimont, 1989). Presently, Flexibacter psychrophilus belongs to the family Cytophagaceae, order Cytophagales and is the etiological agent of BCWD. The remainder of the thesis will use F. psychrophilus when referring to "C. psychrophila."

Flexibacter psychrophilus is a cytophagal bacterium, in a group of bacteria that causes many kinds of fish diseases, (e.g. gill disease, tail rod, peduncle disease, columnaris disease, and cold-water disease). Bullock (1972) examined the similarities and differences between isolates of this bacterium with regard to morphology, physiology, and serology. He reported that the morphological types were different, while the physiological characteristics of the strains from freshwater and estuarine sources were similar. He also reported that 29 out of 55 myxobacterial isolates, including the marine strain, exhibited a serological relationship to F. psychrophilus by agar gel precipitin tests. His results from the slide agglutination test showed that the cultures of the causative agent of columnaris disease agglutinated only with their antiserum, while cross

agglutination was observed between bacteria causing peduncle and cold-water disease with their specific antisera. The cross agglutination provided strong evidence that both diseases were caused by the same pathogen (Bullock, 1972). Holt (1972) used macroscopic tube agglutination and an absorbed serum preparation against six strains of F. psychrophilus. Five of the strains isolated from fish in Oregon, formed the same serological patterns and differed from a sixth strain isolated from fish in New Hampshire. Nevertheless, both groups shared at least one common antigen. About 30 strains of F. psychrophilus isolated from fish in Oregon, Washington, Minnesota, Michigan, Alaska, Idaho, New Hampshire, and British Columbia, Canada gave positive slide agglutination reactions with antiserum against F. psychrophilus strain SH3-81, an Oregon isolate (Holt et al.,1990).

Typical clinical lesions of BCWD have been reported by a number of researchers, Davis (1946), Rucker et al. (1953), Borg (1960), Anderson and Conroy (1969), Conroy and Herman (1970), Bullock and Snieszko (1970), Pacha and Ordal (1970), and Schachte (1983). The disease first appeared as a white discoloration on the tail which then spread over the caudal peduncle until the color changed to a dirty white. At this stage, the sloughing of the tissue was often observed, resulting in exposed muscle and vertebral column. Davis (1946) never observed lesions anterior to the anal fin, because the fish died before the infection could proceed further. Some infected fish had lesions on the isthmus and over the back just anterior to the dorsal fin or showed an abnormally dark colored area with no apparent surface lesions (Borg, 1960). Otis (1984) injected F.

psychrophilus strain N86, which was isolated from a female rainbow trout (O. mykiss) with a severe caudal lesion in Rhode Island, into steelhead trout (Salmo gairdneri). He reported that the branching, swelling, and hemorrhagic skin and muscle were the most obvious lesions followed by hemorrhage of swim bladder, liver, visceral fat, intestine, and peritoneal wall.

Microscopic examination by Davis (1946) of muscular lesions of fingerling rainbow trout infected with BCWD revealed numerous long rod-shaped bacteria and invasion around the muscle fibers by the bacteria, which caused congestion and local hemorrhages. Wood and Yasutake (1956) examined fingerling coho salmon infected with acute BCWD and found the bacterium in a capillary of pseudobranch of the gill, in the heart, peritoneum, spleen, kidney, muscular layer of intestine, air bladder, liver, pancreas, and in the hypodermis and dermis of muscle. Lesions were also found in the mouth, palate, lower jaw, and operculum. The bacterium was found in the trunk kidney, especially in the renal glomeruli, and afferent and efferent glomerular capillaries causing necrosis and degeneration of the tubules. They also observed inflammation and necrosis of heart myofibrils. Lesions of this organ may lead to death in many fish (Wood and Yasutake, 1956).

Borg (1960) tried to cause BCWD by injecting fish with F. psychrophilus and its extracellular products (ECPs). The lesions formed on the fish injected with the live bacterium were similar to those occurring during natural infection. No lesions resulted from the injection of ECPs. He suggested that the pathogenicity of BCWD was due to the presence of the live bacterium and the amount of

ECPs released by bacterium should receive additional study. Otis (1984) injected rainbow trout with F. psychrophilus and produced many clinical signs similar to those of natural infections, including focal interstitial hemorrhage, accumulation of melanin, and cellular proliferation inside Bowman's capsules of the kidney. The bacterium also stimulated the endothelial cells in the atrial and ventricular myocardium and caused congestion of blood vessels in the spleen, focal necrosis of the gastric gland, vacuoles in the cytoplasm of pancreatic acinar cells, and hemorrhage in the visceral fat and swim bladder. The histopathology of liver from infected fish revealed focal dilation, congestion of sinusoids, and atrophy of hepatocytes. He also observed that the injection of ECPs caused clinical signs of BCWD by degrading components of trout skin and muscle, and could lyse red blood cells and had fibrinogenase activity. Because ECPs have the ability to prevent healing, suppress the host's inflammatory response, and inhibit phagocytic activity, they play an important role in the production of cold-water disease (Otis, 1984).

Among the aquatic cytophagal bacteria, only F. psychrophilus, F. columnaris, and F. maritimus are known to be invasive pathogens of fish, while the remainder are considered opportunistic (Bullock, 1972) or saprophytic on the skin (Pacha and Porter, 1968). Cold-water disease is frequently found in juvenile coho salmon, and may affect all species of the salmonids but rarely found in fingerling fall chinook, O. tshawytscha (Amos, 1985). The disease has been reported in North America (Holt, 1987), Europe (Bernardet and Kerouault, 1989), and East Asia (Wakabayashi, 1990). The disease may have been introduced to Europe by contaminated salmonid eggs

from Washington, USA, and may have existed for a long time before being discovered in France (Bernardet and Kerouault, 1989). Wakabayashi (1990) reported the classic signs of BCWD were found in juvenile coho salmon in some hatcheries located in Iwate and Miyagi, Japan. He found four different strains of F. psychrophilus.

Water temperature plays an important role in fish disease, particularly BCWD. Only two other diseases, fin rot, which affects Atlantic salmon, Salmo salar (Schneider and Nicholson, 1980), and cold-water vibriosisis or Hitra disease (Egidius et al. (1986) are often found at lower rather than higher temperatures. Bacterial cold-water disease is rarely found at high temperatures, possibly because of an increase in the hosts' healing ability (Anderson and Roberts, 1975) and/or the decrease of bacterial multiplication at high temperature (Holt, 1987). Cold water is closely associated with epizootics, which usually occur during the spring, when the water temperatures are between 3 and 15°C (Holt, 1987). Borg (1960) and Holt (1987) studied the growth of F. psychrophilus in vitro and reported a similar growth pattern. Temperature permitting bacterial growth ranged from 3 to 25°C with the optimum growth about 15 to 20°C. The shortest generation time was about 4 h at 15°C in tryptone yeast extract broth plus salts medium, while the longest was about 26 h at 3°C (Holt, 1987). Flexibacter psychrophilus produces a disease only within a specific temperature range. By injecting the bacterial cells into large coho salmon, chinook salmon (O. tshawytscha), and rainbow trout, Holt (1989) reported that high mortalities were found in fish held at 3 to 15°C water temperatures, while reduced deaths (near 0%) were found in fish held at 23°C.

Although F. columnaris is in the same genus as F. psychrophilus, its virulence increases with increasing water temperature (Holt et al., 1975).

Transmission of BCWD did not occur even when healthy fish were placed in the aquarium with those exhibiting cold-water disease (Borg, 1960). Borg (1960) did successfully transmit the bacterium by lightly scarifying the caudal peduncle and exposing the fish to F. psychrophilus for 5 min at 10°C. He reported that 12 of 17 sacrificed fish developed classic lesions of cold-water disease within 8 days. The bacterium was reisolated from 11 of the experimental animals. The possible vertical transmission of the bacterium has been reported by Borg (1960) and Holt (1972). Epizootics of cold-water disease occurred at Minter Creek, Dungeness, and Willapa fishery stations (Washington) and may have been transmitted on or in contaminated eggs. These stations obtained eggs from the Green River Hatchery (also in Washington) which also had an epizootic of cold-water disease (Borg, 1960). Holt (1972) reported that BCWD might be transmitted from mature adult coho salmon on or in eggs and then to "susceptible alevins." He found F. psychrophilus in the internal organs and blood of about 50% of mature adult coho salmon returning to the Siletz and Fall Creek Hatcheries in Oregon. From these findings, the eggs may play an important role in transmission of the bacterium from one generation to another. The numbers of bacterial cells found on salmonid eggs have been reported at 10^7 cells/mm^2, of which the majority were Cytophaga and Pseudomonas (Trust, 1972).

Because BCWD causes serious losses during epizootics, many experiments testing both drugs and chemicals have been conducted to develop methods for control of this disease. Amend et al. (1965) mixed both fish-meat and Oregon pellet diets with sulmet (sulfamethazine), S.E.Z. (sulfaethoxy-pyridazine), and gantrisin (sulfisoxazole) and fed these to juvenile coho salmon. They reported that each of these drugs could reduce the mortality caused by BCWD. Gantrisin and S.E.Z. at the rate of 4 gm per 100 pounds of fish per day gave good control. In in vitro studies, gantrisin had four times the bacteriostatic activity of sulmet, was less toxic, and more acceptable to the fish (Amend et al., 1969). Furanace is one of the most effective drugs against bacterial fish pathogens. As little as 1.5 ppm, inhibited four of six cultures of F. psychrophilus and completely inhibited all strains at 3.1 ppm (Ross, 1972). Holt and Conrad (1975) studied furanace both in vitro and in vivo for control of BCWD in young coho salmon. They suggested 0.5 ppm of furanace in a water bath for 1 h every 3 days for fry for a total treatment. Because furanace is easily absorbed by fish, it is useful in controlling diseases of salmonid sac-fry (Holt and Conrad, 1975).

A suggested method of controlling BCWD in salmonids is the treatment of eggs with iodophors (Betadine, or Wescodyne) at 25 ppm for about 1 min (Ross and Smith, 1972) but has been unsuccessful. Immunizing salmonid against BCWD was first studied by Holt (1987). He successfully immunized yearling coho salmon by intraperitoneal injection of formalin-killed F. psychrophilus cells plus Freunds Complete Adjuvant (FCA) with 100% protection, while the unvaccinated control fish had 43% loss after subcutaneous injection

of 8.6 x 10^5 cells/ml of virulent F. psychrophilus. The immersion method used with smaller fish (less than 1 gm) did not give good protection compared to injection of larger fish with FCA plus formalin-killed cells (Holt, 1987). Fish can be immunized against BCWD but when challenged with a highly virulent and large dose of F. psychrophilus, they may still contract the disease (Holt, 1987).

MATERIALS AND METHODS

Bacterial source and maintenance

The Flexibacter psychrophilus strain SH3-81 received from Dr. Richard A. Holt, Chief Fish Pathologist Oregon Department of Fish and Wildlife, Corvallis, Oregon, was used throughout the experiments. The bacterium was originally isolated in 1981 at the Sandy Hatchery, Oregon, from the kidney of a juvenile coho salmon, Oncorhynchus kitsuch, which showed classic signs of BCWD. The lyophilized bacterium was cultured in tryptone yeast extract (TYE) broth medium (Fujihara and Nakatani, 1971) composed of 0.4% tryptone and 0.04% yeast extract adjusted to 7.1-7.3 pH. The viability examination via a wet mount showed a long rod that was motile by gliding. The bacterium formed a fried egg-like colony on Cytophaga agar (CA) (Anacker and Ordal, 1959) composed of 0.05% tryptone, 0.02% beef extract, 0.02% sodium acetate, 0.05% yeast extract, and 1% bacto-agar adjusted to 7.1-7.3 pH. The stock cultures were maintained in tubes containing 4 ml CA with 0.15% bacto-agar added. The stock cultures were incubated at 14-16°C for 3 days to allow for rapid multiplication of the bacterial cells. They were then kept at 4°C. The stock cultures were transferred every 1-2 months.

Growth in different media

The bacterium was cultured in six different broth media; tryptone yeast infusion (TYI) (Pacha and Ordal, 1967) composed of 0.4% tryptone and 3% yeast infusion; tryptone yeast extract plus salt (TYES) (Holt, 1987) composed of 0.4% tryptone, 0.04% yeast extract,

0.05% magnesium sulfate ($7H_2O$) and 0.05% calcium chloride ($2H_2O$); tryptone yeast extract (TYE) (Garnjobst, 1945) composed of 0.4% tryptone and 0.04% yeast extract; Liewes medium (Liewes et al., 1982) composed of 0.45% casitone, 1.0% gelatin, 0.0015% sodium acetate, 0.45% yeast extract, 0.204% sodium chloride, 0.012% potassium chloride, 0.003% potassium phosphate monobasic, 0.006% magnesium sulfate ($7H_2O$), and 0.004% calcium chloride ($2H_2O$); Cytophaga medium composed of 0.05% tryptone, 0.02% beef extract, 0.02% sodium acetate, 0.05% yeast extract, and 1% bacto-agar; Myxo-medium (Ordal and Rucker, 1944) composed of 0.5% tryptone. All six media were adjusted to 7.1-7.3 pH by using 0.5 and 0.1 N sodium hydroxide. After three passages of F. psychrophilus in each medium at 14-16°C, they were calibrated to 0.15 absorbance at a wave-length 525 nm on a spectrophotometer (Spectronic 20, Bausch and Lomb), then one ml of each culture medium was transferred into duplicate Nephelo culture flasks (Belco Glass, Inc., Vineland, NJ) containing 50 ml of the same broth media. All flasks were placed on an orbital shaker (Queue orbital shaker model 4710) at 130 rpm setting and 14-16°C incubation. The data were recorded as percent transmittance every 3-4 hours for 3 days, then they were converted to absorbance by the equation Abs=2-log%T. The growth curves on the six media were plotted between time and average optical density.

At the maximum growth curve or stationary phase of the bacterium in each medium, twenty ml from each culture flask were transferred to a 50 ml centrifuge tube and centrifuged at 3000 rpm for 15 min by using an IEC HN-SII Centrifuge, Damon/IEC Division,

then the bacterial cells were weighed and the average maximum wet weight in mg/ml determined.

Growth at different shaking rates and temperatures

The two media which yielded the highest optical density and wet weight, TYI and TYES, were selected for growth studies. Growth was examined at shaking rates of 70, 100, and 130 rpm and incubation temperatures of 15±1°C and 18±2°C. These experiments were the same as the previous growth experiments except for the shaking rates and incubation temperatures. The bacterial growth in both broth media was compared by plotting absorbance versus time. Tryptone yeast infusion medium was chosen as the culture medium for the following experiments because it supported the best growth in these experiments.

Viable cell counts

Viable cell counts of F. psychrophilus cultured in TYI broth at a shaking rate of 130 rpm and an incubation temperature of 15±1°C for 2 days were performed by the spread plate method. The broth culture was centrifuged at an angular velocity of 3000 rev/min at 17°C for 15 min in a Sorvall RC-5B Refrigerated Superspeed Centrifuge (Du Pont Instrument) and washed 3 times with phosphate buffered saline (PBS). The bacterial cells were diluted with PBS to obtain five concentrations. Each bacterial concentration was subjected to a ten-fold dilution. One-tenth ml of medium from each dilution was dropped onto the surface of three modified Cytophaga agar (MCA) plates (Wakabayashi and Egusa, 1980), composed of 0.2%

sodium acetate, 0.02% calcium chloride, and 1.5% agar adjusted to a pH of 7.1. The dilution was spread with a glass rod dipped in ethanol and flame sterilized, then incubated at 15±1°C for 3-4 days. The culture plates were counted on a darkfield colony counter, and 30-300 colonies were counted in order to calculate the average number of bacterial cells per milliliter for each optical density reading. The viable cell counts per milliliter were plotted against optical density.

Medium lethal dose (LD_{50})

Experimental fish

The fingerling coho salmon were held at the Oregon State University Fish Disease Laboratory (OSU FDL) in aquaria supplied with continuous flow of pathogen-free water at a temperature of 12±0.5°C. The fish were fed with Oregon Moist Pellet.

Preliminary test

A preliminary test was performed to determine the range of bacterial dilutions which cause 0% and 100% mortality of coho salmon. After three passages of virulent F. psychrophilus through coho salmon, the virulent bacterium was prepared at 10^9, 10^7, and 10^5 cells/ml dilutions. A 0.05 ml dose of each dilution was injected intramuscularly into five coho salmon with a 1 ml disposable tuberculin syringe and a 26 gauge needle. At 10 days after injection, 100% mortality was found in the group of fish receiving the 10^9 cells/ml dilution, while all the fish at the lowest dilution survived. The dilutions between 10^5 and 10^9 cells/ml were used to determine the LD_{50}.

Definitive test

A total of 120 coho salmon with an average weight of 21.8±4.0 gm were randomly transferred from the stock aquaria into six 68 L experimental aquaria (twenty fish in each aquarium). The fish were acclimated in the aquaria for 7 days with 1 L/min continuous water flow system at 12°C and fed daily. Five dilutions of virulent bacteria were calibrated to between 10^5 and 10^9 cells/ml, using the curve of viable cell counts as reference. A dose of 0.05 ml of each dilution was injected intramuscularly into 20 fish in each aquaria using a 1 ml disposable tuberculin syringe and a 26 gauge needle. The twenty fish in the sixth aquarium were injected intramuscularly with 0.05 ml of PBS. The fish were fed daily. The number of dead fish was recorded and the bacterium was isolated from muscle and kidney lesions on a CA plate. After 4 days at an incubation temperature of 14-16°C, F. psychrophilus formed a fried egg colony. The experiment terminated when there was no fish mortality for 3 days. The percent of accumulated mortality of each dilution was plotted on a probability 4 log cycles graph. The medium lethal dose was calculated according to Litchfield and Wilcoxon (1949).

Hematologic study

A total of 150 coho salmon, mean weight 25.6±6.3 gm, were transferred randomly from stock aquaria into six 68 liters experimental aquaria (twenty-five fish in each aquaria). The fish were acclimated in the aquaria for 7 days with a 1 L/min continuous water flow system at 12°C and were fed daily. Two bacterial concentrations, 2.5 x 10^7 and 9.4 x 10^5 cells/ml, were selected from

the LD_{50} experiment and prepared using the curve of viable cell counts as reference. The high concentration was selected from the concentration which caused high mortality but still allowed enough fish to collected throughout the experiment. The low concentration was selected from the concentration which caused low mortality. A 0.05 ml dose of each dilution was injected into the muscle just posterior to the dorsal fin above lateral line of 50 fish from two aquaria, while the control group was injected with 0.05 ml PBS.

The blood parameters, hematocrit, red blood cell, white blood cell, plasma glucose, total plasma protein, and hemoglobin were collected and analyzed according to Wedemeyer and Yasutake (1977), except as stated. The data were collected on 1, 4, 7, 10, 13, and 19 day(s) after the injection or challenges. On each collecting day, six fish were randomly removed from each of the high concentration, low concentration, and control group. The fish were immobilized within 30 seconds using benzocaine before cutting the body just posterior to the anal opening and anterior to the caudal peduncle. The blood was drawn from caudal blood vessels by using a heparinized hematocrit capillary. Three to four blood capillaries were enough to measure all parameters.

A few small drops from the first blood capillary were smeared on clean glass slides, then the blood was drawn by a red blood cell diluting pipet to mark 1 and then diluted to mark 101 (1:100 dilution) with Yokoyama's white cell fluid (Conroy and Herman, 1970). After mixing well, a few drops of diluted blood were blown out, and the rest was transferred into a 2.0 ml SlickSeal micro-centrifuge tube (Island Scientific, Bainbridge Island, WA). The tubes

of the diluted blood were kept in the refrigerator and counted within 2 days after collection. Twenty-five µl of blood was drawn from the first blood capillary by micropipet then transferred into a test tube containing 4 ml of hemoglobin determined reagent. The remaining blood capillaries were sealed at one end with critoseal and centrifuged for 10 min by micro hematocrit centrifuge (IEC MB Centrifuge, Damon/IEC Division). The hematocrit was determined by the hematocrit reader. Next 25 µl blood plasma was drawn by micropipet and transferred into a tube containing 3 ml of total plasma protein determined reagent. The remaining 25 µl of plasma was transferred into a tube containing 3.5 ml of glucose determined reagent. All the determined reagents were prepared accoeding to Wedemayer and Yasutake (1977).

After color of hemoglobin, plasma glucose, and total plasma protein had been developed in the tubes, their percent transmittance were read on the spectrophotometer at wave-length 540, 635, and 540 nm, respectively. The percent transmittance were converted to absorbance by using the previous equation. The absorbance of these parameters was converted to concentration by comparing them with their standard concentration curves. The ranges of standard curves were 12.5-200 mg/dl for plasma glucose, 2-10 gm/dl for total plasma protein, and 4.50-11.25 gm/dl for hemoglobin, which prepared according to Wedemayer and Yasutake (1977).

The tubes of diluted blood were held at room temperature and mixed by using a Vortex mixer before counting red and white blood cells on the hemacytometer under the light microscope using Cartwright's (1968) method. The red blood cells were counted in five

groups of 0.2 x 0.2 mm^3. Each group contained 16 small squares. The counting volume of each group was 0.004 mm^3. The red blood cell dilution was 1/100. The red blood cells were oval shaped, biconvex, and had spherically eccentric nuclei and a pink to rose stain.

$$\text{RBC } (\text{cells}/mm^3) = \frac{\text{Number of RBC counted x dilution}}{0.004 \text{ x Number of groups counted}}$$

$$\text{or} = \text{Number of RBC counted x } 5000$$

The white blood cells were counted in 5 groups of 1 x 1 mm^2. Each group contained 16 large squares. The counting volume of each group was 0.1 mm^3. The white blood cell dilution was 1/100. The white blood cells were spherical or elongate in shape and had light pink to light blue stained cytoplasm.

$$\text{WBC } (\text{cells}/mm^3) = \frac{\text{Number of WBC counted x dilution}}{0.1 \text{ x Number of groups counted}}$$

$$\text{or} = \text{Number of WBC counted x } 200$$

The concentration of hemoglobin, number of red blood cells, and percent hematocrit of each fish sampling were used to calculate the mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) by the equation described in Diagnostic Laboratory Hematology by Cartwright (1968) with minor modifications.

$$\text{MCV } (\mu m^3/\text{cell}) = \frac{\text{Hematocrit (\%) x 10}}{\text{RBC (x } 10^6 \text{ cells/mm}^3)}$$

$$\text{MCH (pg/cell)} = \frac{\text{Hemoglobin (gm/dl) x 10}}{\text{RBC (x } 10^6 \text{ cells/mm}^3)}$$

$$\text{MCHC (\%)} = \frac{\text{Hemoglobin (gm/dl) x 100}}{\text{Hct (\%)}}$$

The blood smear slides were stained with modified Giemsa (May-Grunwald) stain for blood smears (Barbara and Howard, 1979). The slides were counted for the percent of lymphocyte, neutrophil, thrombocyte, and monocyte. A total of one hundred cells of white blood cells were counted under the light microscope. The criteria for differentiating the types of white blood cells (lymphocyte, neutrophil, thrombocyte, and monocyte) were based on Ellis (1977), Wedemeyer and Yasutake (1977), Yasutake and Wales (1983), and Rowley (1990). The percent of each type of white blood cell was calculated an absolute cell count (cells/mm^3).

Histopathologic Study

Paraffin embedding preparation

After the blood was drawn from the fish for hematology studies, the fish were examined for external and internal gross lesions. The fish were cut at the operculum, opened at the abdomen, and preserved for at least 24 h in 10% buffer formalin solution, which consisted of 100 ml distilled water, 4.0 gm monosodium phosphate, 6.5 gm di-sodium phosphate, and 900 ml formaldehyde.. The volume of fixative reagent was about 20 times greater than the volume of the fish to avoid the postmortem changes of the tissues. Humason (1972) procedure for embedding tissue in paraffin was

used. The organs were cut into small pieces and placed in cassettes. The cassettes of bone containing tissue were decalcified in the decalcifying solution (Decalcifying solution CS 510-10, Fisher Scientific) for 24-48 h. The remaining fixative and decalcifying reagents in the tissue were washed out by running tap water over them for 2 h. The cassettes were left overnight in a 75% ethanol bath. The cassettes were passed through one bath of 85% ethanol, three baths of 95% ethanol, three baths of 100% ethanol, one bath of equal mixtures of 100% ethanol and xylene, three baths of xylene, and two baths of 60°C paraffin. These steps were controlled by an automatic tissue processor (Fisher Tissuematon). The embedded tissues in the cassettes were removed and placed in a block and then covered with paraffin. The blocks of embedded tissues were cut 6 μm thick (3 μm as design) with an American Optical 820 Spencer Microtome. The slices of tissues were stretched on the 45°C water bath, then picked up using glass slides and labeled with diamond-tip marker. The slides were left overnight on the 45°C slide warmer for firm adhesion. Five naturally BCWD-infected coho salmon were also examined histologically. These fish were collected during the BCWD epizootic in July of 1989 at the Oxbow Hatchery, Oregon (collected by Terry D. Kreps, Fish Pathologist, Clackamas fish pathology laboratory, Clackamas, Oregon).

Tissue staining procedures

The tissue slides were routinely stained with hematoxylin and eosin stain (H&E) according to Humason (1972). The selected tissue slides were stained with May-Grunwald giemsa stain for paraffin

sections according to Yasutake and Wales (1983), modified from the method of Humason (1972). The slides were covered with cover-slice, sealed with cytoseal 60 mounting medium (Stephens Scientific, Denville, NJ), and examined under a light-microscope. The data were recorded as the number of fish with histopathologic changes in each tissue.

Statistical analysis

The LD_{50} was calculated at a 95% confidence interval according to Litchfield and Wilcoxon (1949). The values of hematocrit, red blood cell counts, white blood cell counts, glucose, total plasma protein, hemoglobin, MCV, MCH and MCHC were analyzed by using one-way analysis of Variance (ANOVA). This analysis was applied to each group of the bacterial dilutions and controls as well as to the samples counted on the specified day after injection. When a significant difference within groups was found, Tukey's multiple comparison procedure was applied. The values of lymphocyte, neutrophil, thrombocyte, and monocyte were analyzed using the distribution-free ANOVA or Kruskal-Wallis Test, because the data did not fit the basic assumptions of one-way ANOVA as closely as the Kruskal-Wallis Test. The significant difference results in each group were also analyzed by Tukey's multiple comparison procedure. The exception was the Wilcoxen Rank Sum Test which was used to compare samples on the specified day after injection.

The values of gross lesions and microscopic lesions were also analyzed by using Kruskal-Wallis Test and Tukey's multiple comparison procedure. All statistical analysis, except the LD_{50}

calculation, was done using Statgraphics version 3.0 of Graphic Software System, Inc., IBM personal computer XT, and the text Statistics of Devore and Peck (1986).

Note: The data from the higher bacterial concentration injection, 2.5 x 10^7 cells/ml, were eliminated from all 4 types of white blood cells because of the difficulty in counting from blood smears of the severe disease condition. Before using ANOVA, each data group was subjected to plot Multiple Box and Whisker Plot. The extreme outliers, which altered the analysis were removed so that the number of observations was not equal in every group. The criteria for determining the significant difference between means was a 95% confidence interval or P=0.05.

RESULTS

Flexibacter psychrophilus strain SH3-81 is a slender, rod shaped, gram-negative bacterium, about 0.3 to 0.5 μm in width and 2 to 7 μm in length (Figure 1). On CA, after 4-5 days incubation at 15±1°C, the bacterium grows only on the surface and exhibits a fried egg-like colony, which is glossy and convex at the center with a thin spreading transparent margin (Figure 2). The fried egg-like colony becomes transparent and less raised with a longer incubation time. The colony morphology on the enriched modified Cytophaga agar was smooth, convex with a regular edge, and cream to yellow color after 3-4 days incubation. Gliding motility was observed by the wet mount method under a 1000x light-microscope, a technique suggested by Holt et al. (1990). With strain SH3-81, the gliding motility was also observed with the hanging drop method but hard to observed with other strains reported by Bernardet and Kerouault (1989).

The gliding motility varied with the incubation temperature. When cultured in TYES and TYI broth media, and incubated at 12, 13, and 14°C the percentages of bacterial cells that were motile by means of gliding were about 0%, 50%, and 95%, respectively. The bacterium in TYES and TYI broth culture at an incubation temperature of 12 to 13°C and a shaking rate of 70 rpm formed a tiny clump of cells 24 hours after inoculation. The clump of bacterial cells increased in size with incubation time. A low incubation temperature seemed to alter the gliding ability and caused clumping of these bacterial cells.

Figure 1. Flexibacter psychrophilus strain SH3-81 after incubation for 1-2 days in TYE broth culture shows a slender, rod shaped, ~0.3 to 0.5 μm in width and 2 to 7 μm in length. (Crystal violet stain, bar=10 μm)

Figure 2. Flexibacter psychrophilus strain SH3-81 exhibits a fried egg-like colony, glossy convex at the center with a thin spreading margin, on Cytophaga agar after 4-5 days incubation at 15±1°C. (bar=0.25 cm)

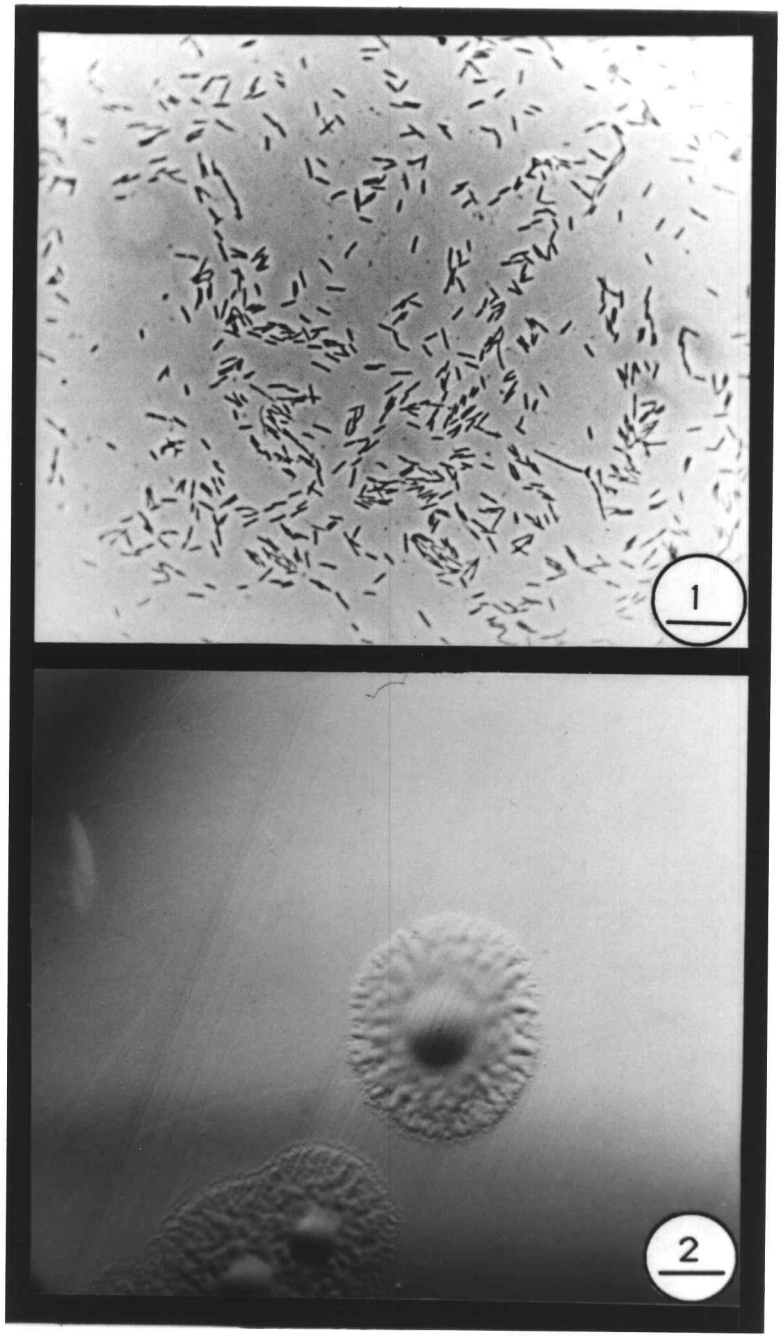

Figure 1 and 2

Growth in different media

Flexibacter psychrophilus strain SH3-81's ability to grow in the different media, TYI, TYES, TYE, CA, Liewes media, and myxo-medium varies (Figure 3). The Cytophaga medium supported only limited growth of the bacteria, while the remaining media gave good growth. The Liewes medium, TYES, and TYI gave higher growth and optical density reading than the others. The TYI and TYES media seemed to give a higher wet weight of bacterial cells at the stationary phase than TYE, Liewes, and myxo-medium (Figure 4). The wet weight of bacterial cells from the Cytophaga medium could not be measured due to the low cell concentration. The better growth of the bacterium in TYES over TYE was also found by Holt (1987), who developed the TYES salt containing medium. Liewes medium was the other salt containing medium and gave the highest optical reading but did not give the highest wet weight. Flexibacter psychrophilus can grow well even in a simple broth medium of myxo-medium containing only 0.5% tryptone. Both TYI and TYES seem to be the best media for bacterial mass cell culture because of their high optical readings and maximum wet weight at the stationary phase. The TYI and TYES broth media were chosen as the culture media for the next experiment.

Growth at different shaking rates and temperatures

The bacterial growth was measured at an optical density reading of 525 nm versus time. Their growth in both TYI and TYES were similar at 70, 100, and 130 rpm shaking rates, but the higher shaking rate seemed to give better growth (Figure 5 and 6).

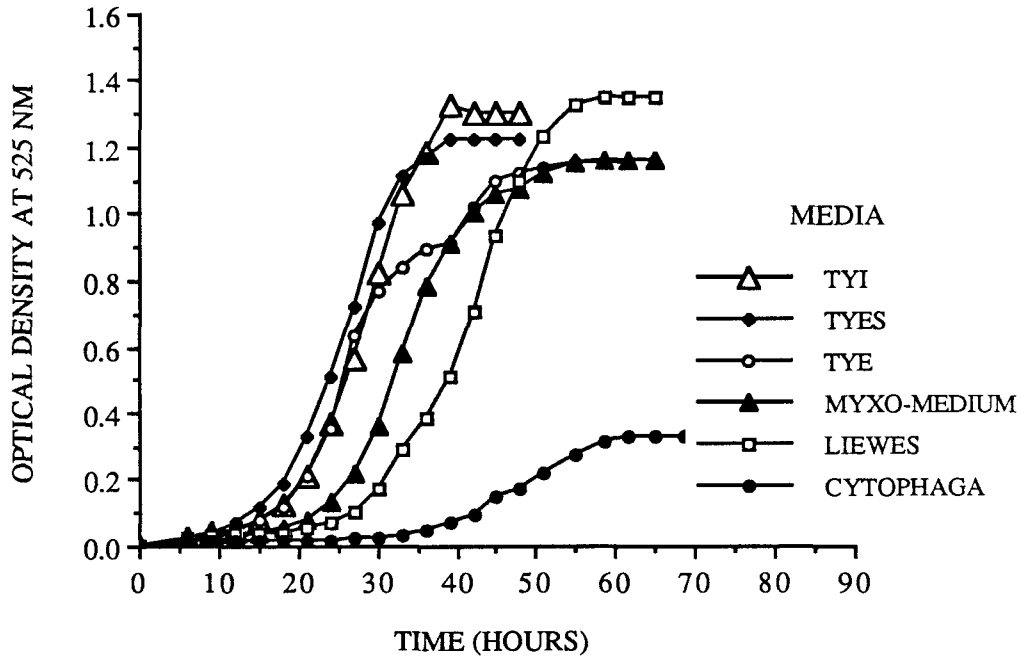

Figure 3. Growth of Flexibacter psychrophilus isolate SH3-81 culture in selected broth media at 15±1°C and shaken at 130 rpm on an orbital shaker.

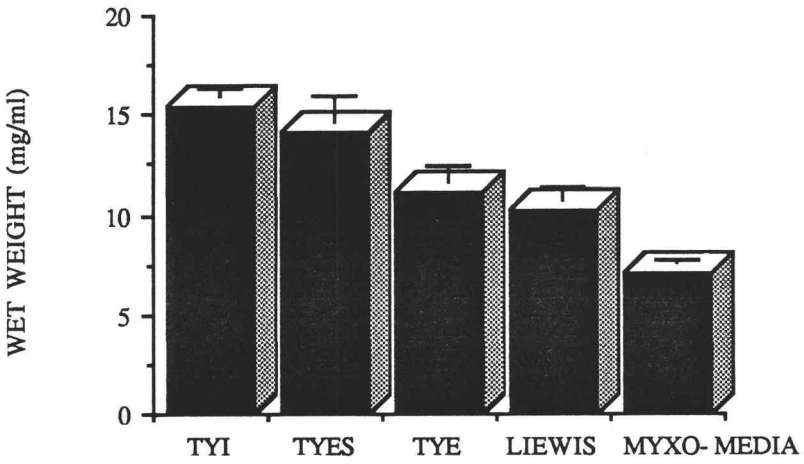

Figure 4. Maximum wet weight (mean±SE) of Flexibacter psychrophilus cells from selected broth media centrifuged and weighed at the stationary phase of the culture from Figure 1.

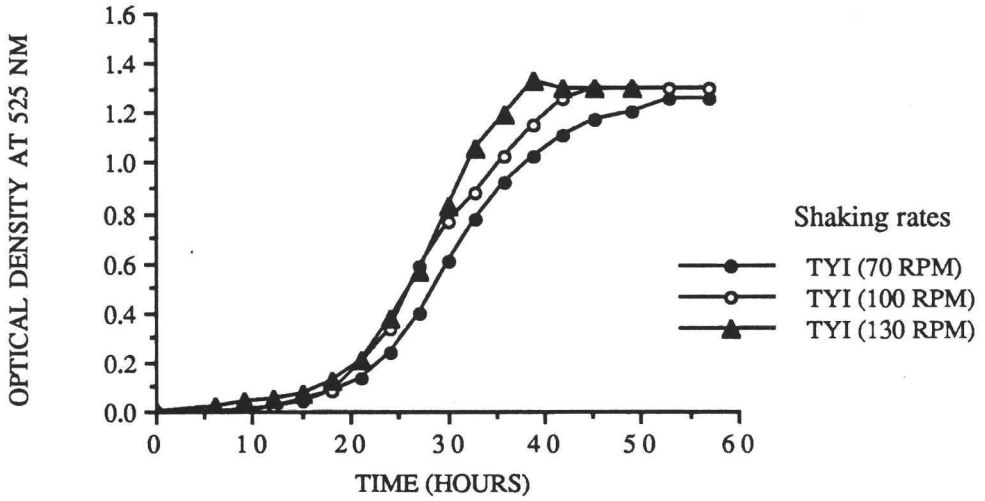

Figure 5. Growth of Flexibacter psychrophilus in tryptone yeast infusion (TYI) broth comparing different shaking rates when incubated at 15±1°C.

The difference was most apparent when the growth at 130 rpm was compared to 70 rpm. The growth curve at the 130 rpm shaking rate seemed to be faster than the others but leveled off at about the same stationary phase for TYI and a little greater for TYES.

At two different incubation temperatures, 15±1°C and 18±2°C, both TYI and TYE media gave better bacterial growth at the higher incubation temperature (Figure 7). The bacterial culture in TYES seemed to grow faster than TYI at log phase but have a lower optical reading than TYI at the stationary phase of both incubation temperatures. Because of the high wet weight and optical density reading of the bacterial culture in TYI, it was used in all the remaining experiments.

Viable cell counts

The triplicate spread plate method was performed to determine the concentration of F. psychrophilus at different optical density readings (Figure 8). This growth curve was used as the reference for bacterial concentration at selected optical density readings for the LD_{50}, hematologic, and histopathologic experiments.

Medium lethal dose (LD_{50})

The LD50 experiment was used to indicate the virulence of F. psychrophilus and for selecting the bacterial concentration for hematological and histopathological examination of infected fish. Bacterial dilutions were prepared at 9.5×10^5, 6.6×10^6, 5.8×10^7, 2.1×10^8, 5.3×10^8 cells/ml. Five days after injection, dead coho salmon were found at the highest injection dose (Table 1).

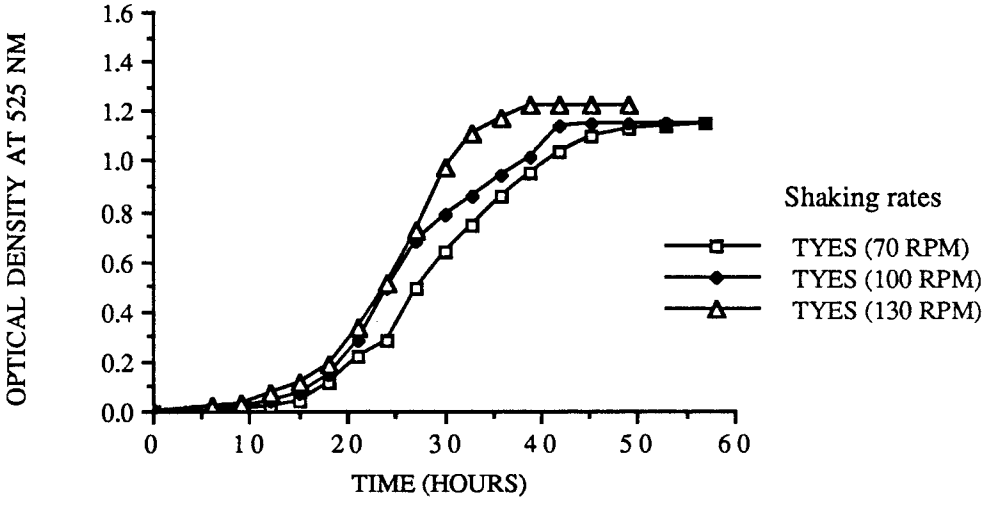

Figure 6. Growth of Flexibacter psychrophilus in tryptone yeast extract plus salts (TYES) broth at different shaking rates and incubated at 15±1°C.

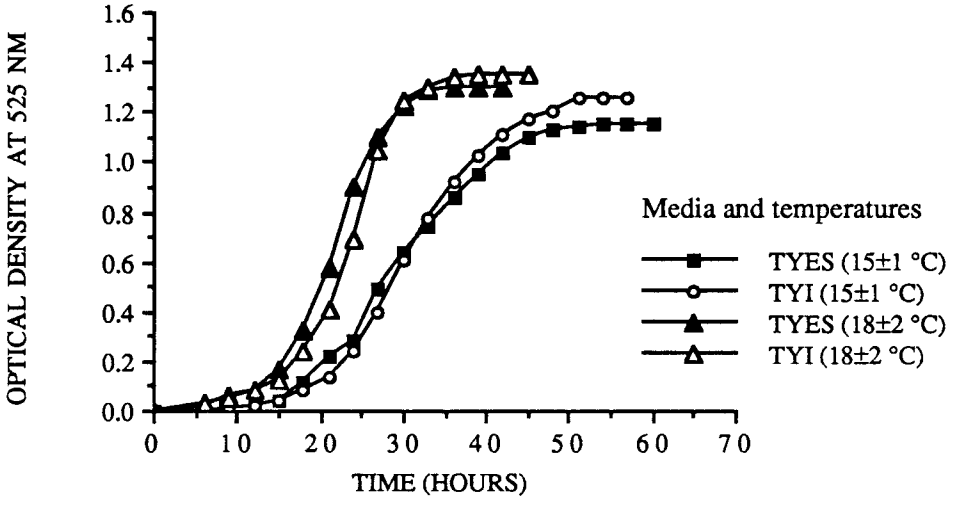

Figure 7. Comparison of the growth of Flexibacter psychrophilus isolate SH3-81 culture in TYI and TYES broth when incubated at 15±1 or 18±2°C.

Many dead coho salmon were observed on days 6, 7, and 8 in all bacterial dilutions but not in the PBS control. The accumulated mortality of each group was plotted against the injection dose (Figure 9). The LD_{50} of F. psychrophilus strain SH3-81 injected intramuscularly in 21.8 gm juvenile coho salmon was ~2.3 x 10^7 cells/ml after 15 days (Appendix 1). The range of LD_{50} at a 95% confidence interval was between 8.5 x 10^6 and 6.2 x 10^7 cells/ml calculated by the method of Litchfield and Wilcoxon (1949).

The response of experimental coho salmon began a few minutes after injection with the development of a black patchy color at the site of injection. The black patchy area has reduced in size by the next day and was hardly observed in the PBS control fish. The skin began to swell on the third day and after the fifth day a black pigment surrounding the lesion was observed. In the severe disease condition, the skin and muscle lesion was open and branch. Some coho salmon died before the swollen lesion became an open lesion. Localized hemorrhaging liver was observed in dead coho salmon (Table 2). The bacterium was reisolated on CA medium from kidney and muscle tissue of dead coho salmon that still had red gills and clear corneas. Flexibacter psychrophilus was recovered from 100% of the muscle isolations and 90% of the kidney isolations from the dead coho salmon. Holt (1987) also recovered the bacterium in over 90% of the kidney isolations from dead coho salmon from the LD_{50} experiment. Three days after the experiment, the bacterium was recovered in only 9% of the muscle isolations and 2% of the kidney isolations from the 47 surviving coho salmon.

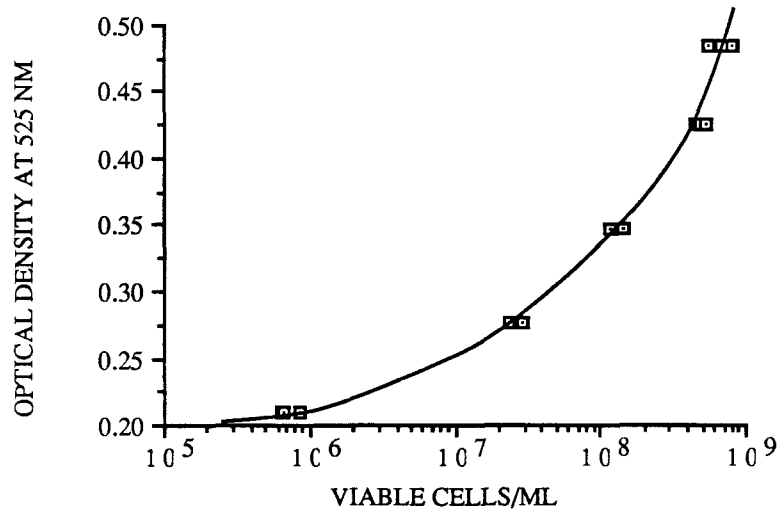

Figure 8. Correlation of Flexibacter psychrophilus isolate SH3-81 viable cells versus optical density (525 nm) in TYI broth measured by the spread plate method.

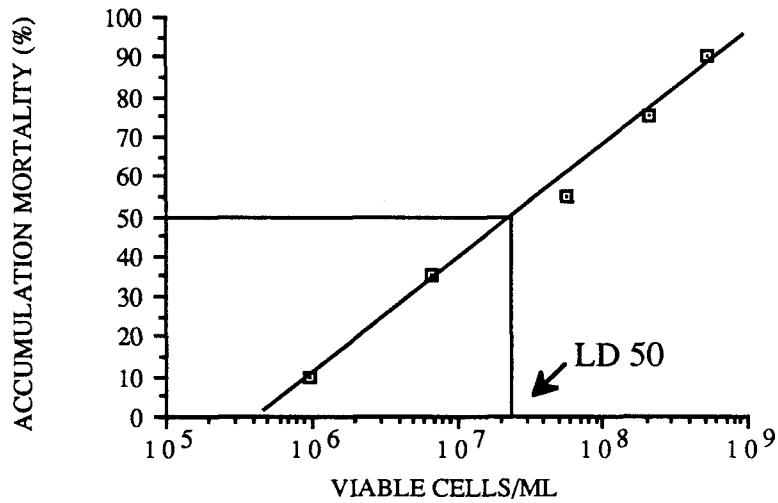

Figure 9. Mortality of coho salmon (~21.8 gm) when given an intramuscular injection of five concentrations of Flexibacter psychrophilus cells. The LD_{50} after 15 days was 2.3×10^7 cells/ml.

Table 1. Cumulative mortality of coho salmon[a] caused by intramuscular injection of five concentrations of Flexibacter psychrophilus cells. The LD_{50} after 15 days was 2.3×10^7 (8.5×10^6 to 6.2×10^7) cells/ml at a 95% confidence interval.[b]

Day(s)	Viable cells injected (cells/ml)					Control
	9.5×10^5	6.6×10^6	5.8×10^7	2.1×10^8	5.3×10^8	PBS
0	0	0	0	0	0	0
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	0	0
4	0	0	0	0	0	0
5	0	0	0	0	2	0
6	2	0	2	1	8	0
7	2	4	6	7	12	0
8	2	7	8	9	14	0
9	2	7	11	13	16	0
10	2	7	11	15	16	0
11	2	7	11	15	17	0
12	2	7	11	15	17	0
13	2	7	11	15	18	0
14	2	7	11	15	18	0
15	2	7	11	15	18	0
Total mortality	2/20	7/20	11/20	15/20	18/20	0/20
% mortality	10	35	55	75	90	0

a Fish were ~21.8 gm in weight and held at 12±0.5°C. The volume of the injection was 0.05 ml/fish.

b The LD_{50} was calculated according to Litchfield and Wilcoxon (1949).

Table 2. Number of coho salmon with gross lesions and Flexibacter psychrophilus present in the dead fish from LD_{50} experiment.

Characteristics	No. of fish from 29 observations
1. External gross lesions	
isthmus petechia hemorrhage	1
pectoral fin hemorrhage	1
pelvic fin hemorrhage	1
anal fin hemorrhage	1
gill hemorrhage	1
skin swelling (soft and bloody tissue inside)	17
skin blanching (with soft and bloody tissue)	12
2. Internal gross lesions	
liver petechia hemorrhage	1
liver focal hemorrhage	15
kidney bloody swelling	5
spleen bloody swelling	3
swim bladder hemorrhage	2
visceral fat hemorrhage	1
small intestine hemorrhage	6
no changes	4
3. F. psychrophilus present [a]	
muscle	29
kidney	26

[a] An unidentified gram-negative short rod was found with F. psychrophilus in the muscle of two coho salmon but the quantity was low. Flexibacter psychrophilus was found in the muscle of 4 fish and muscle and kidney of one fish out of 47 fish that survived for 3 days after LD_{50} experiment.

Hematologic study

There is no report of hematologic changes in bacterial cold-water disease fish. Usually, septicemic fish pathogens cause obvious hematological changes. The report of Waagbø et al. (1988) showed that Vibrio salmonicida caused a decrease in red blood cells, hemoglobin, hematocrit, and mean corpuscular volume (MCV) in Atlantic salmon (Salmo salar L.). The experiments were conducted to determine the changes of some hematologic parameters during a sampling period of 1, 4, 7, 10, 13, and 19 day(s) after the intramuscular injection of two concentrations of F. psychrophilus, at 9.4×10^5 and 2.5×10^7 cells/ml, and a PBS control. Samplings up to day 4 were considered as the early disease condition (showing a small swollen skin). Sampling at days 7 through 10 were considered as the severe disease condition (showing a large swollen and/or opened skin). Sampling at days 13 through 19 were considered as the recovery condition (showing a healed wound). The severe disease condition of the lower and the higher bacterial infection groups were found at day 7 and days 7 and 10, respectively.

Hematocrit or packed cell volume (Hct)

The hematocrit of both the control and lower bacterial infection groups showed no significantly different means during the sampling period (Table 3), while the higher bacterial injection group had a significant difference ($P=0.0320$). The Hct increased during the early development of the lesion in the higher bacterial injection at 4 days and decreased in the severe lesion at day 10. When infected coho salmon began to recover, the Hct increased and by the end of the

Table 3. Hematocrits of intramuscularly F. psychrophilus-injected coho salmon[a] compare with PBS-injected control. Values are expressed as mean (% packed red blood cell volume) ± SE. The parentheses show the number of observations. The bold numbers indicate data collected in the severe disease condition.

Day(s) after injection	Viable cells injected (cells/ml)			Sig. level
	PBS Control	9.4×10^5	2.5×10^7	
1	44.7 ± 1.5 (5)	43.7 ± 1.8 (6)	34.0 ± 1.3 [b] (6)	n.s.[f]
4	43.5 ± 2.5 (6)	47.7 ± 2.7 (6)	42.9± 3.0 [c] (6)	n.s.
7	39.6 ± 1.7 (6)	**41.3 ± 1.8** (6)	**41.6 ± 4.1** [b,c] (5)	n.s.
10	[e]39.9 ± 1.1 (6)	[e]44.0 ± 3.0 (6)	[d]**31.0 ± 2.3** [b] (6)	p=0.0032
13	41.1 ± 1.0 (6)	44.1 ± 3.3 (6)	42.8 ± 3.4 [c] (6)	n.s.
19	39.2 ± 0.28 (6)	38.0 ± 1.6 (6)	39.3 ± 1.5 [b] (6)	n.s.
Sig. level	n.s.	n.s.	p=0.0320	

a Fish were ~25.6 gm in weight and held at 12±0.5°C. The volume of injection was 0.05 ml/fish.

b,c These letters stand for a statistical summary of the column. Values with the same letter do not have significantly different means of hematocrit. (b<c)

d,e These letters stand for a statistical summary of the row. Values with the same letter do not have significantly different means of hematocrit. (d<e)

f The means are not significantly different. (n.s.=not significant)

experiment has returned to the same level as the control group. The lowest Hct, 31.0%, was found at day 10 and was significantly lower than the Hct of 39.9% found in the control group (P=0.0032).

Red blood cell (RBC)

The change in RBC counts in the infected fish was similar to the change in hematocrit. Red blood cell counts in both the control and the lower bacterial infection groups showed no significant difference in means during the sampling period (Table 4), while the higher bacterial injection group was significantly different (P=0.0213). In the severe disease condition of infected coho salmon, the RBC counts dropped to 1.06 x 10^6 cells/mm^3 at day 7, while RBC counts in the early recovering coho salmon was increased to 1.56 x 10^6 cells/mm^3 at day 13. Only days 7 and 10, did the control and bacterial injection groups show significantly different means of RBC counts with P=0.0432 and P=0.0207, respectively.

White blood cell (WBC)

The BCWD-infected coho salmon showed highly significant differences in the level of WBC after challenges during the sampling period. The significance level was as little as 0.0006 in the lower bacterial injection and even lower than 0.0001 in the higher bacterial injection (Table 5). The low levels of WBC were found in the most severe condition of the higher bacterial infection group on days 7 and 10. Both bacterial injection groups had markedly increased levels of WBC, 14.2 x 10^3 and 15.3 x 10^3 cells/mm^3, on the early recovery date, which then decreased to the same level as the control

Table 4. Red blood cell counts in intramuscularly F. psychrophilus-injected coho salmon[a] compare with PBS-injected control. Values are expressed as mean (RBC x $10^6/mm^3$) ± SE. The parentheses show the number of.observations. The bold numbers indicate data collected in the severe disease condition.

Day(s) after injection	Viable cells injected (cells/ml)			Sig. level
	PBS Control	9.4×10^5	2.5×10^7	
1	1.52 ± 0.03 (9)	1.59 ± 0.05 (6)	1.45 ± 0.09 [b,c] (6)	n.s.[h]
4	1.46 ± 0.09 (6)	1.38 ± 0.08 (6)	1.47 ± 0.15 [b,c] (6)	n.s.
7	[e]1.51 ± 0.11 (6)	[d,e]**1.37 ± 0.12** (6)	[d]**1.06 ± 0.12** [b] (6)	p=0.0432
10	[f,g]1.49 ± 0.05 (6)	[g]1.61 ± 0.10 (6)	[f]**1.22 ± 0.10** [b,c] (6)	p=0.0207
13	1.65 ± 0.17 (6)	1.69 ± 0.07 (6)	1.56 ± 0.12 [c] (6)	n.s.
19	1.43 ± 0.06 (6)	1.44 ± 0.12 (6)	1.54 ± 0.08 [b,c] (6)	n.s.
Sig. level	n.s.	n.s.	p=0.0213	

[a] Fish were ~25.6 gm in weight and held at 12±0.5°C. The volume of injection was 0.05 ml/fish.

[b,c] These letters stand for a statistical summary of the column. Values with the same letter do not have significantly different means of RBC. (b<c)

[d,e,f,g] These letters stand for a statistical summary in the row. Values with the same letter do not have significantly different means of RBC. (d<e and f<g)

[h] The means are not significantly different. (n.s.=not significant)

Table 5. White blood cell counts in intramuscularly F. psychrophilus-injected coho salmon[a] compare with PBS-injected control. Values are expressed as mean (WBC x $10^3/mm^3$) ± SE. The parentheses show the number of observations. The bold numbers indicate data collected in the severe disease condition.

Day(s) after injection	Viable cells injected (cells/ml) PBS Control	9.4×10^5	2.5×10^7	Sig. level
1	9.2 ± 2.2 (6)	9.1 ± 1.1 [b,c] (6)	9.9 ± 1.1 [g] (6)	n.s.[n]
4	10.2 ± 1.4 (6)	7.1 ± 1.2 [b] (6)	9.2 ± 1.6 [g] (6)	n.s.
7	[j]10.9 ± 0.9 (6)	[j]**8.9 ± 1.3** [b,c] (5)	[i]**3.8 ± 1.2** [e,f] (6)	p=0.0012
10	[l]9.7 ± 0.8 (6)	[m]14.2 ± 0.9 [d] (6)	[k]**2.6 ± 0.9** [e] (6)	p<0.0001
13	13.2 ± 1.0 (6)	13.1 ± 1.7 [c,d] (5)	15.3 ± 1.5 [h] (6)	n.s.
19	10.9 ± 1.0 (6)	9.2 ± 0.3 [b,c] (6)	7.9 ± 0.8 [f,g] (6)	n.s.
Sig. level	n.s.	p=0.0006	p<0.0001	

[a] Fish were ~25.6 gm in weight and held at 12±0.5°C. The volume of injection was 0.05 ml/fish.

[b,c,d,e,f,g,h] These letters stand for a statistical summary of the column. Values with the same letter do not have significantly different means of WBC. (b<c<d and e<f<g<h)

[i,j,k,l,m] These letters stand for a statistical summary of the row. Values with the same letter do not have significantly different means of WBC. (i<j and k<l<m)

[n] The means are not significantly different. (n.s.=not significant)

group by the end of the experiment. The higher bacterial infection of coho salmon caused the lowest WBC counts, 3.8 x 10^3 cells/mm^3, among the three experimental groups (P=0.0012).

Plasma glucose

Some changes of plasma glucose concentration in BCWD-infected coho salmon were found in this study (Table 6). The higher bacterial injection caused significantly different means (P=0.0031). The severe disease condition caused an increase in plasma glucose up to 139.3 mg/dl, followed by a decrease to the same concentration as the control group by day 13. Before the severe disease condition developed in the lower bacterial injection group, the plasma glucose concentration was significantly less than the control group (P=0.0324). The variation of plasma glucose concentration seems to depend on the concentration of the injected bacteria and the disease condition of BCWD.

Total plasma protein (TPP)

The coho salmon infected from the higher bacterial injection had significantly different means of TPP during the sampling period (P=0.0004, Table 7). The total plasma protein decreased to 1.37 and 1.25 gm/dl in the severe disease condition, but were significantly lower than the control group (P=0.0092 at day 7 and P=0.0030 at day 10). The total plasma protein increased markedly from 1.25 gm/dl to 4.42 gm/dl in the early stage of recovery. The BCWD in coho salmon decreased the concentration of TPP.

Table 6. Plasma glucose of intramuscularly F. psychrophilus-injected coho salmon[a] compare with PBS-injected control. Values are expressed as mean (mg/dl) ± SE. The parentheses show the number of observations. The bold numbers indicate data collected in the severe disease condition.

Day(s) after injection	Viable cells injected (cells/ml)			Sig. level
	PBS Control	9.4×10^5	2.5×10^7	
1	127.9 ± 3.9 (3)	99.8 ± 24.2 (6)	97.0 ± 7.6 [b,c] (6)	n.s.[f]
4	[e]121.1 ± 4.0 (6)	[d]80.8± 20.3 (5)	[d,e]119.5 ± 14.8 [b,c] (6)	p=0.032
7	107.7 ± 11.4 (6)	**114.4 ± 24.4** (6)	**139.3 ± 6.8** [c] (5)	n.s.
10	115.5 ± 6.7 (6)	101.0 ± 24.5 (6)	**127.9± 14.7** [b,c] (4)	n.s.
13	99.8 ± 8.8 (6)	86.4 ± 8.7 (6)	81.9 ± 3.7 [b] (6)	n.s.
19	90.2 ± 9.2 (5)	85.3 ± 10.3 (6)	86.5± 12.9 [b] (5)	n.s.
Sig. level	n.s.	n.s.	p=0.003	

a Fish were ~25.6 gm in weight and held at 12±0.5°C. The volume of injection was 0.05 ml/fish.

b,c These letters stand for a statistical summary of the column. Values with the same letter do not have significantly different means of plasma glucose (b<c).

d,e These letters stand for a statistical summary of the row. Values with the same letter do not have significantly different means of plasma glucose. (d<e.)

f The means are not significantly different. (n.s.=not significant)

Table 7. Total plasma protein of intramuscularly F. psychrophilus-injected coho salmon[a] compare with PBS-injected control. Values are expressed as mean (gm/dl) ± SE. The parentheses show the number of observations. The bold numbers indicate data collected in the severe disease condition.

Day(s) after injection	Viable cells injected (cells/ml)			Sig. level
	PBS Control	9.4×10^5	2.5×10^7	
1	3.48 ± 0.19 (6)	3.55 ± 0.16 (6)	3.00 ± 0.21 [b,c,d] (6)	n.s.[i]
4	3.51 ± 0.34 (6)	3.33 ± 0.48 (4)	2.48 ± 0.44 [b,c,d] (6)	n.s.
7	[f] 3.83 ± 0.22 (6)	[e,f] **2.72 ± 0.75** (6)	[e] **1.37 ± 0.60** [b,c] (5)	p=0.0092
10	[h] 4.00 ± 0.171 (6)	[h] 4.54 ± 0.76 (6)	[g] **1.25 ± 0.68** [b] (6)	p=0.0030
13	3.43 ± 0.14 (6)	4.74 ± 0.57 (6)	4.42 ± 0.51 [d] (6)	n.s.
19	3.91 ± 0.271 (6)	4.09 ± 0.33 (6)	3.31 ± 0.29 [c,d] (6)	n.s.
Sig. level	n,s,	n,s,	p=0.0004	

a Fish were ~25.6 gm in weight and held at 12±0.5°C. The volume of injection was 0.05 ml/fish.

b,c,d These letters stand for a statistical summary of the column. Values with the same letter do not have significantly different means of total plasma protein. (b<c<d)

e,f,g,h These letters stand for a statistical summary of the row. Values with the same letter do not have significantly different means of total plasma protein. (e<f and g<h)

i The means are not significantly different. (n.s.=not significant)

Hemoglobin (Hb)

Only the higher bacterial injection caused significantly different means of Hb during the sampling period (P=0.0002, Table 8). When the severe condition developed, the Hb decreased to as low as 8.23 gm/dl. The Hb significantly increased in the recovery stage at days 13 and 19 but remained at the same level in the control group. The concentration of Hb in the higher bacterial injection group was significantly lower than the other groups by day 10 (P=0.0007).

Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), and Mean Corpuscular Hemoglobin Concentration (MCHC)

The blood parameters, MCV, MCH, and MCHC, were calculated from Hct, Hb, and RBC in each sampling of coho salmon. The fish infected with BCWD showed significantly different means for MCV in both the lower and higher bacterial injection groups (P=0.0013 and P=0.0104) during the sampling period (Table 9). The MCV of the lower bacterial infection group at day 4 was 347.0 μm^3/cell, which was significantly higher than the other groups (P=0.0174). At the severe disease condition at day 7, the MCV of the coho salmon infected with 2.5 x 10^7 cells/ml of F. psychrophilus significantly increased from 292.0 to 368.0 μm^3/cell. There was one slightly significant difference (P=0.0494) in the means of MCH between the experimental groups at day 7 (Table 10). The MCH 83.0 pg/cell of infected coho salmon was greater than the MCH 68.0 pg/cell of the control group.

Table 8. Hemoglobin of intramuscularly F. psychrophilus-injected coho salmon[a] compare with PBS-injected control. Values are expressed as mean (gm/dl) ± SE. The parentheses show the number of observations. The bold numbers indicate data collected in the severe disease condition.

Day(s) after injection	Viable cells injected (cells/ml)			Sig. level
	PBS Control	9.4×10^5	2.5×10^7	
1	11.16 ± 0.24 (10)	10.99 ± 0.49 (6)	10.24 ± 0.32 [b,c,d] (6)	n.s.[g]
4	11.21 ± 0.62 (5)	9.73 ± 0.77 (5)	10.82 ± 0.58 [c,d] (6)	n.s.
7	10.03 ± 0.38 (6)	**10.01 ± 0.41** (6)	**8.66 ± 0.84** [b,c] (6)	n.s.
10	[f]9.96 ± 0.30 (6)	[f]10.73 ± 0.32 (6)	[e]**8.23 ± 0.46** [b] (6)	p=0.0007
13	11.13 ± 0.48 (6)	12.16 ± 0.53 (6)	11.92 ± 0.57 [d] (6)	n.s.
19	10.58 ± 0.17 (6)	10.75 ± 0.71 (6)	11.46 ± 0.45 [d] (6)	n.s.
Sig. level	n.s.	n.s.	p=0.0002	

a Fish were ~25.6 gm in weight and held at 12±0.5°C. The volume of injection was 0.05 ml/fish.

b,c,d These letters stand for a statistical summary of the column. Values with the same letter do not have significantly different means of hemoglobin. (b<c<d)

e,f These letters stand for a statistical summary of the row. Values with the same letter do not have significantly different means of hemoglobin. (e<f)

g The means are not significantly different. (n.s.=not significant)

Table 9. Mean corpuscular volume (MCV) of intramuscularly F. psychrophilus-injected coho salmon[a] compare with PBS-injected control. Values are expressed as mean (μm^3/cell) ± SE. The parentheses show the number of observations. The bold numbers indicate data collected in the severe disease condition.

Day(s) after injection	Viable cells injection (cells/ml)			Sig. level
	PBS Control	9.4×10^5	2.5×10^7	
1	290 ± 13 (5)	276 ± 15 [b] (6)	278 ± 10 [d] (6)	n.s.[h]
4	[f]299 ± 11 (6)	[g]347 ± 11 [c] (6)	[f]292 ± 16 [d,e] (5)	p=0.0174
7	268 ± 19 (6)	**310 ± 17** [b,c] (6)	**368 ± 44** [e] (5)	n.s.
10	269 ± 10 (6)	275 ± 14 [b] (6)	**259 ± 15** [d] (6)	n.s.
13	260 ± 15 (6)	261 ± 14 [b] (6)	274 ± 14 [d] (6)	n.s.
19	277 ± 13 (6)	268 ± 13 [b] (6)	264 ± 18 [d] (6)	n.s.
Sig. level	n.s.	p=0.0013	p=0.0104	

[a] Fish were ~25.6 gm in weight and held at 12±0.5°C. The volume of injection was 0.05 ml/fish.

[b,c,d,e] These letters stand for a statistical summary of the column. Values with the same letter do not have significantly different means of MCV. (b<c and d<e)

[f,g] These letters stand for a statistical summary of the row. Values with the same letter do not have significantly different means of MCV. (f<g)

[h] The means are not significantly different. (n.s.=not significant)

Table 10. Mean corpuscular hemoglobin (MCH) of intramuscularly F. psychrophilus-injected coho salmon[a] compare with PBS-injected control. Values are expressed as mean (pg/cell) ± SE. The parentheses show the number of observations. The bold numbers indicate data collected in the severe disease condition.

Day(s) after injection	Viable cells injected (cells/ml)			Sig. level
	PBS Control	9.4×10^5	2.5×10^7	
1	74 ± 2	69 ± 4	72 ± 4	n.s.[d]
	(5)	(6)	(6)	
4	76 ± 3	73 ± 7	72 ± 3	n.s.
	(5)	(5)	(6)	
7	[b]68 ± 3	[b,c]**75 ± 4**	[c]**83 ± 5**	p=0.0494
	(6)	(6)	(6)	
10	67 ± 2	67 ± 4	**69 ± 3**	n.s.
	(6)	(6)	(6)	
13	70 ± 5	71 ± 3	77 ± 2	n.s.
	(6)	(6)	(6)	
19	75 ± 3	75 ± 3	75 ± 4	n.s.
	(6)	(6)	(6)	
Sig. level	n.s.	n.s.	n.s.	

a Fish were ~25.6 gm in weight and held at 12±0.5°C. The volume of injection was 0.05 ml/fish.

b,c These letters stand for a statistical summary of the row. Values with the same letter do not have significantly different means of MCH. (b<c)

d The means are not significantly different. (n.s.=not significant)

Flexibacter psychrophilus caused an increase in Hb concentration and in the size of a single red cell of coho salmon. The increase of Hb in a single cell was not enough to increase Hb in the whole blood because of the low numbers of RBC and percent packed cell volume. There was some evidence that the percent (or gm/dl) of MCHC decreased in coho salmon infected with BCWD (Table 11). During the sampling period, both bacterial injection groups had significantly different means of MCHC (P=0.0087 and P=0.0018), while the control group did not. After the severe disease condition, the MCHC increased to the same concentration as the control group. In the severe disease condition of the higher bacterial injection group, the MCHC decreased to 23.4% and during the recovery increased significantly to about 28%. The MCHC of the lower bacterial injection group was considerably lower than that of the control group (P=0.0318).

Differential count of white blood cell

The differential count of WBC was used to compare the coho salmon infected with 9.4×10^5 cells/ml of F. psychrophilus and the control group. There were significant changes in the mean number of lymphocytes in the infected coho salmon during the sampling period (P=0.0060, Table 12). The absolute count of lymphocytes in early recovery condition at day 10 increased significantly to 9.0×10^3 cells/mm^3, which was twice as many as the control group (P=0.0453). The percent of lymphocytes increased significantly to 70% in the recovery condition at day 13 (P=0.0360).

Table 11. Mean corpuscular hemoglobin concentration (MCHC) of intramuscularly F. psychrophilus-injected coho salmon[a] compare with PBS-injected control. Values are expressed as mean (%) ± SE. The parentheses show the number of observations. The bold numbers indicate data collected in the severe disease condition.

Day(s) after injection	Viable cells injection (cells/ml)			Sig. level
	PBS Control	9.4×10^5	2.5×10^7	
1	25.8 ± 0.6 (5)	25.2 ± 0.6 [b,c] (6)	25.7 ± 0.7 [d,e] (6)	n.s.[h]
4	[g]26.1 ± 0.6 (5)	[f]23.1 ± 0.9 [b] (4)	[f,g]23.7 ± 0.8 [d] (5)	p=0.0318
7	25.4 ± 0.8 (6)	**24.3 ± 0.8** [b,c] (6)	**23.4 ± 1.6** [d] (5)	n.s.
10	24.9 ± 0.7 (6)	24.4 ± 1.3 [b,c] (6)	**26.8 ± 0.9** [d,e] (6)	n.s.
13	27.0 ± 0.7 (6)	27.5 ± 1.0 [b,c] (6)	28.2 ± 0.8 [e] (6)	n.s.
19	27.1 ± 0.6 (6)	28.2 ± 1.1 [c] (6)	28.6 ± 0.9 [e] (6)	n.s.
Sig. level	n.s.	p=0.0087	p=0.0018	

a Fish were ~25.6 gm in weight and held at 12±0.5°C. The volume of injection was 0.05 ml/fish.

b,c,d,e These letters stand for a statistical summary of the column. Values with the same letter do not have significantly different means of MCHC. (b<c and d< e)

f,g These letters stand for a statistical summary of the row. Values with the same letter do not have significantly different means of MCHC. (f<g)

h The means are not significantly different. (n.s.=not significant)

Table 12. Lymphocytes of intramuscularly F. psychrophilus-injected coho salmon[a] compare with PBS-injected control. Values are expressed as mean ± SE. The parentheses show the number of observations. The bold numbers indicate data collected in the severe disease condition.

	Lymphocytes (%)			Lymphocytes (x 10^3 cells/mm^3)		
Day(s) after injection	Control PBS	Injection dose 9.4 x 10^5 (cells/ml)	Sig. level	Control PBS	Injection dose 9.4 x 10^5 (cells/ml)	Sig. level
1	50.7 ± 4.5 (6)	45.0 ± 9.2 (6)	n.s.	4.3 ± 0.7 (6)	4.5 ± 1.1 [b] (6)	n.s.[d]
4	51.5 ± 4.6 (6)	61.2 ± 6.0 (6)	n.s.	5.4 ± 1.2 (6)	4.1 ± 0.6 [b] (6)	n.s.
7	46.3 ± 5.1 (6)	**40.4 ± 7.2** (5)	n.s.	5.2 ± 0.8 (6)	**3.8 ± 0.9** [b] (5)	n.s.
10	47.5 ± 7.0 (6)	62.3 ± 8.0 (6)	n.s.	4.5 ± 0.5 (6)	9.0 ± 1.4 [c] (6)	p=0.0453
13	42.0 ± 9.3 (6)	70.0 ± 5.4 (6)	p=0.0360	5.5 ± 1.2 (6)	9.3 ± 1.6 [c] (5)	n.s.
19	62.7 ± 1.5 (6)	65.0 ± 6.3 (6)	n.s.	6.9 ± 0.6 (6)	5.6 ± 0.5 [b,c] (6)	n.s.
Sig. level	n.s.	n.s.		n.s.	p=0.0060	

a Fish were ~25.6 gm in weight and held at 12±0.5°C. The volume of injection was 0.05 ml/fish.

b,c These letters stand for a statistical summary of the column. Values with the same letter do not have significantly different means of absolute lymphocyte count. (b<c)

d There is insufficient evidence from this small amount of data to indicate the different means of percent and absolute lymphocyte counts. (n.s.=not significant)

The percent and absolute count of neutrophils were quite different in the infected coho salmon during the sampling period with P=0.0223 and P=0.0239, respectively (Table 13). The same changes in infected coho salmon were found in both percent and absolute count of neutrophils. Both counts increased at day 10 and decreased to the normal level of the control by day 19. The neutrophils increased in number and percent only at the stage of recovery from BCWD. The number of neutrophils in infected coho salmon increased to three times greater than the control group at day 10 (P=0.0453). The only change in thrombocytes was found in the severe disease condition on day 7 (Table 14). The thrombocytes of infected coho salmon were 51.2%, which was greater than the control group, which had 37.0% thrombocytes.

Monocytes are usually found in low numbers in peripheral blood circulation and exhibit a limited phagocytic activity. Their activity can be increased when transformed to macrophages (Roberts, 1978). The percent and absolute count of monocytes in both infected and control coho salmon were not found to have any significant differences within their groups during the sampling period (Table 15). Interestingly, at the early disease condition, monocytes of infected coho salmon had significantly lower percentage (P=0.0177) and absolute counts (P=0.0124). The decrease of monocytes in peripheral blood circulation seemed to be localized in the damaged tissue of the skeletal muscle (as shown later in the histopathologic study).

Table 13. Neutrophils of intramuscularly F. psychrophilus-injected coho salmon[a] compare with PBS-injected control. Values are expressed as mean ± SE. The parentheses show the number of observations. The bold numbers indicate data collected in the severe disease condition.

	Neutrophils (%)			Neutrophils (x 10^3 cells/mm^3)		
Day(s) after injection	Control PBS	Injection dose 9.4×10^5 (cells/ml)	Sig. level	Control PBS	Injection dose 9.4×10^5 (cells/ml)	Sig. level
1	12.0 ± 1.5 (6)	8.0 ± 2.3 [b,c] (6)	n.s.	1.0 ± 0.2 (6)	0.7 ± 0.3 [d,e] (5)	n.s.[f]
4	14.8 ± 5.4 (6)	7.7 ± 0.8 [b,c] (6)	n.s.	1.6 ± 0.5 (6)	0.6 ± 0.1 [d,e] (6)	n.s.
7	9.8 ± 1.5 (6)	**5.6 ± 2.4** [b,c] (5)	n.s.	1.1 ± 0.2 (6)	**0.5 ± 0.3** [d,e] (5)	n.s.
10	4.5 ± 2.0 (6)	9.3 ± 2.3 [c] (6)	n.s.	0.4 ± 0.2 (6)	1.3 ± 0.2 [e] (6)	p=0.0453
13	9.8 ± 4.2 (6)	3.7 ± 0.8 [b,c] (6)	n.s.	1.2 ± 0.5 (6)	0.4 ± 0.1 [d,e] (5)	n.s.
19	3.2 ± 0.8 (6)	1.7 ± 1.0 [b] (6)	n.s.	0.4 ± 0.1 (6)	0.2 ± 0.1 [d] (6)	n.s.
Sig. level	n.s.	p=0.0223		n.s.	p=0.0239	

a Fish were ~25.6 gm in weight and held at 12±0.5°C. The volume of injection was 0.05 ml/fish.

b,c,d,e These letters stand for a statistical summary of the column. Values with the same letter do not have significantly different means of percent and absolute neutrophil counts. (b<c and d<e)

f There is insufficient evidence from this small amount of data to indicate the different means of percent and absolute neutrophil counts. (n.s.=not significant)

Table 14. Thrombocytes of intramuscularly F. psychrophilus-injected coho salmon[a] compare with PBS-injected control. Values are expressed as mean ± SE. The parentheses show the number of observations. The bold numbers indicate data collected in the severe disease condition.

Day(s) after injection	Thrombocytes (%) Control PBS	Thrombocytes (%) Injection dose 9.4×10^5 (cells/ml)	Sig. level	Thrombocytes ($\times 10^3$ cells/mm^3) Control PBS	Thrombocytes ($\times 10^3$ cells/mm^3) Injection dose 9.4×10^5 (cells/ml)	Sig. level
1	31.3 ± 4.2 (6)	35.0 ± 9.8 (6)	n.s.	3.1 ± 0.9 (6)	2.7 ± 0.5 (6)	n,s,[b]
4	27.3 ± 6.6 (6)	30.5 ± 5.5 (6)	n.s.	2.6 ± 0.5 (6)	2.3 ± 0.8 (6)	n.s.
7	37.0 ± 3.8 (6)	**51.2 ± 5.5** (5)	p=0.0358	3.9 ± 0.3 (6)	**4.3 ± 0.4** (5)	n.s.
10	43.5 ± 5.2 (6)	22.0 ± 7.8 (6)	n.s.	4.3 ± 0.7 (6)	3.2 ± 1.1 (6)	n.s.
13	45.0 ± 7.7 (6)	23.3 ± 5.8 (6)	n.s.	6.1 ± 1.4 (6)	3.0 ± 1.1 (5)	n.s.
19	30.5 ± 1.8 (6)	29.3 ± 6.2 (6)	n.s.	3.4 ± 0.4 (6)	2.8 ± 0.6 (6)	n.s.
Sig. level	n.s.	n.s.		n.s.	n.s.	

a Fish were ~25.6 gm in weight and held at 12±0.5°C. The volume of injection was 0.05 ml/fish.

b There is insufficient evidence from this small amount of data to indicate the different means percent and absolute thrombocyte counts. (n.s.=not significant)

Table 15. Monocytes of intramuscularly F. psychrophilus-injected coho salmon[a] compare with PBS-injected control. Values are expressed as mean ± SE. The parentheses show the number of observations. The bold numbers indicate data collected in the severe disease condition.

	Monocytes (%)			Monocytes (x 10^3 cells/mm^3)		
Day(s) after injection	Control PBS	Injection dose 9.4×10^5 (cells/ml)	Sig. level	Control PBS	Injection dose 9.4×10^5 (cells/ml)	Sig. level
1	6.0 ± 3.1 (6)	8.0 ± 2.2 (6)	n.s.	0.78 ± 0.59 (6)	0.66 ± 0.14 (6)	n.s [b]
4	6.0 ± 2.1 (6)	1.0 ± 0.6 (6)	p=0.0177	0.58 ± 0.18 (6)	0.08 ± 0.05 (6)	p=0.0124
7	6.7 ± 2.2 (6)	**2.8 ± 1.5** (5)	n.s.	0.69 ± 0.21 (6)	**0.26 ± 0.16** (5)	n.s.
10	4.3 ± 1.2 (6)	6.3 ± 2.2 (6)	n.s.	0.46 ± 0.15 (6)	0.81 ± 0.25 (6)	n.s.
13	3.0 ± 1.6 (6)	3.3 ± 1.6 (6)	n.s.	0.41 ± 0.20 (6)	0.33 ± 0.51 (5)	n.s.
19	3.7 ± 0.8 (6)	4.0 ± 1.9 (6)	n.s.	0.38 ± 0.06 (6)	0.36 ± 0.17 (6)	n.s.
Sig. level	n.s.	n.s.		n.s.	n.s.	

a Fish were ~25.6 gm in weight and held at 12±0.5°C. The volume of injection was 0.05 ml/fish.

b There is insufficient evidence from this small amount of data to indicate the different means of percent and absolute monocyte counts. (n.s.=not significant)

Histopathologic study

Using the same samples as the hematologic study, a histopathologic study defined the changes in intramuscularly F. psychrophilus- and PBS-injected coho salmon on days 4, 7, and 19. A histopathologic study was also conducted on naturally BCWD-infected coho salmon.

Gross lesions

The number of coho salmon with gross lesions was significantly different between the injected groups on day 7 (Table 16). The development of the gross lesions was similar to those of the LD_{50} study. A few minutes after being challenged with PBS and F. psychrophilus, the experimental coho salmon developed a black patchy area about 1 to 1.5 cm in diameter at the site of injection. The black patchy area turned grey and decreased in size by the next day and was difficult to detect in the PBS control coho salmon. Two of three coho salmon from the high bacterial injection group developed swollen skin at the site of injection on day 4, while only one coho salmon injected with the low bacterial concentration had swollen skin. The infected coho salmon began to lose their appetite 3 days after injection. In the severe disease condition on day 7, only a few infected coho salmon per aquarium ate any food. The swollen skin, which contained soft and bloody tissue, developed into a severe lesion by day 7 in both bacterial injection groups but the high bacterial injection group had larger lesions which were still visible at day 10. The severe lesion was recognized as a crater-like lesion with

Table 16. Number of coho salmon[a] with gross lesions after intramuscular injection with 2.5 x 10^7 (high dose) and 9.4 x 10^5 (low dose) cells/ml of F. psychrophilus compare with PBS-injected and naturally BCWD-infected[b] coho salmon.

Days after injection	4 Days			7 Days			19 Days			Natural
Injection doses (cells/ml)	high	low	PBS	high	low	PBS	high	low	PBS	Infection
Number of examined fish	3	3	3	5	5	5	3	3	3	5
1. External gross lesions										
gill local redness	0	0	0	1	0	0	0	0	0	0
skin swelling	2	1	0	5	5	2	0	0	0	1
skin blanching	0	0	0	5	5	0	0	0	0	2
caudal fin erosion	0	0	0	0	0	0	0	0	0	1
2. Internal gross lesion										
liver										
petechial hemorrhage	0	0	0	2	1	0	0	0	0	0
focal hemorrhage	1	0	0	1	1	0	0	0	0	3
visceral fat hemorrhage	0	0	0	0	0	0	0	0	0	1
swim bladder hemorrhage	0	0	0	0	0	0	0	0	0	1
kidney bloody swelling	0	0	0	1	0	0	1	0	0	1
spleen bloody swelling	0	0	0	1	0	0	0	0	0	0
Significant level		n.s.			p=0.0457[c]			n.s.		

a Fish were ~25.6 gm in weight and held at 12±0.5°C. The volume of injection was 0.05 ml/fish.

b Fish were ~4.4 gm in weight and collected from the Oxbow Hatchery, OR by Terry D. Kreps, Fish Pathologist, Clackamas fish pathology laboratory, Clackamas, OR.

c There were not enough data to show the significantly different means in the group 7 days after injection by Tukey's multiple ranges test. The significant level of the Kruskal-Wallis test was 0.0457.

an area 0.8 to 3 cm^2 within the open area surrounded by swollen skin.

Two of five coho salmon in the PBS control group also had swollen skin on day 7 but they were smaller in size and lacked soft and bloody tissue. In the severe disease condition, some infected coho salmon developed redness in the gill, a petechial to focal hemorrhage, and bloody swelling in the spleen and posterior kidney. The number of infected coho salmon with gross lesions was significantly different between experimental groups on day 7 (P=0.0457). The coho salmon infected with F. psychrophilus seemed to have more gross lesions than the PBS control group. Two coho salmon in the high bacterial injection group died on day 9 of the experiment.

After the infected coho salmon had passed the severe disease condition, their appetite increased and the lesions began to heal on days 10 and 13 in the low and high bacterial injection groups, respectively. One of three coho salmon in the high bacterial injection group had a bloody swelling of the posterior kidney on day 19.

The coho salmon infected naturally with BCWD had some similarities to those infected experimentally but the pathology, were less severe in the muscle and more severe in the internal organs. The external lesions had a crater-like lesion with less swelling and were located in the area between the posterior dorsal fin and caudal peduncle. Decay of the caudal fin was also found. Hemorrhagic lesions were observed in the liver, visceral fat, and swim bladder. Table 16 shows the number of coho salmon with these lesions.

Microscopic lesions

The number of coho salmon with microscopic lesions was significantly different between the injected groups (Table 17). In early disease condition on day 4, the coho salmon with the high bacterial injection had significantly more histopathologic damages than did the control coho salmon (P=0.0205). In the severe disease condition of BCWD on day 7, both high and low bacterial injection groups had significantly more histopathologic changes than the control coho salmon (P=0.0055). Even though the gross lesions were rarely observed on day 4, microscopic observations found many histologic changes in the tissue of infected coho salmon as compared with the control coho salmon.

The severely diseased tissue of coho salmon infected with F. psychrophilus changed during the course of the experiment. The gills were markedly affected in both the high and low bacterial injection groups in the severe disease condition on day 7. The lesions found on the secondary gill lamellae were hypertrophied (the size of the cells was increased), hyperplastic (the number of cells was increased), and edematous (the amount of water in the cells increased). All naturally infected and 80% of the experimentally infected coho salmon showed hypertrophy of secondary gill lamellae, which could have altered the oxygen- and ion-exchange and resulted in hypoxia. A hemorrhaged gill was found in one coho salmon of the high injection group (Figure 10, 11).

The histopathologic changes were readily observed in the skeletal muscle at the site of infection. At the sampling period on

Table 17. Number of coho salmon[a] with microscopic lesions resulting from intramuscular injection with 2.5×10^7 (high dose) and 9.4×10^5 (low dose) cells/ml of F. psychrophilus compare with PBS-injected and naturally BCWD-infected[b] coho salmon.

Days after injection	4 Days			7 Days			19 Days			Natural
Injection doses (cells/ml)	high	low	PBS	high	low	PBS	high	low	PBS	Infection
Number of examined fish	3	3	3	5	5	5	3	3	3	5
1. Gill										
hypertrophy	0	1	0	3	5	1	2	1	0	5
hyperplasia	2	1	1	2	2	2	0	2	2	0
edema	0	2	0	3	3	1	2	3	0	1
hemorrhage	0	0	0	1	0	0	0	0	0	0
2. Skeletal muscle										
blood capillary invasion	3	3	3	5	5	5	0	0	0	2
leukocytic infiltration	3	3	3	5	5	5	0	0	0	2
bacteria present	3	3	0	5	5	0	0	0	0	2
mixed inflammation	3	3	0	5	5	5	0	0	0	3
focal hemorrhage	3	1	0	5	5	1	0	0	0	2
focal necrosis	3	1	0	5	5	2	0	0	0	3
myofibril regeneration	0	0	0	0	0	0	3	3	3	0
myofibril atrophy	0	0	0	5	5	2	0	0	0	2
3. Liver										
vacuolar degeneration	1	1	1	2	2	1	0	1	1	1
pycnotic nuclei	3	3	0	4	3	0	0	1	0	4
congestion	0	0	0	2	1	0	0	0	0	0
hemorrhage	1	0	0	1	0	0	0	0	0	3
4. Visceral fat										
hemorrhage	0	0	0	0	0	0	0	0	0	2

Table 17. (cont.)

Days after injection	4 Days			7 Days			19 Days			Natural
Injection doses (cells/ml)	high	low	PBS	high	low	PBS	high	low	PBS	Infection
Number of examined fish	3	3	3	5	5	5	3	3	3	5
5. Swim bladder										
hemorrhage	0	0	0	0	0	0	0	0	0	1
6. Spleen[c]										
congestion	0	0	0	1	0	0	0	0	0	0
ellipsoidal necrosis	3	0	0	5	3	0	1	0	0	3
↑ melanomacrophage	0	0	0	1	0	0	1	0	0	2
vacuolation	0	0	0	1	0	0	0	0	0	1
7. Anterior kidney[c]										
↑ melanomacrophage	1	1	0	3	3	1	1	0	0	3
↓ hemopoictic tissue	1	1	0	5	4	1	0	0	0	4
reticuloendothelial necrosis	0	0	0	0	0	0	0	0	0	2
8. Posterior kidney[c]										
renal tubules necrosis	2	3	1	4	4	1	3	1	1	4
congestion	0	0	0	1	0	0	2	1	0	2
↑ melanomacrophage	1	1	1	3	3	0	2	1	1	2
renal tubules thickening	0	0	0	0	0	0	0	0	0	1
9. Glomerulus										
necrosis	0	0	0	0	0	0	0	0	0	1
thickening	0	0	0	0	0	0	0	0	0	1
10. Pancreas										
acinar cells necrosis	0	0	0	3	2	1	0	0	0	0
islets of langerhans necrosis	0	0	0	2	0	0	0	0	0	1
cloudy swelling	0	0	0	0	1	0	0	0	0	1

Table 17. (cont.)

Days after injection	4 Days			7 Days			19 Days			Natural
Injection doses (cells/ml)	high	low	PBS	high	low	PBS	high	low	PBS	Infection
Number of examined fish	3	3	3	5	5	5	3	3	3	5
11. Heart (ventricle)[c]										
focal vacuolation	0	1	0	0	1	0	0	1	0	0
↑ endocardial macrophage	0	1	0	0	1	0	0	0	0	0
swelling and sloughing of endocardium	0	0	0	0	0	0	0	0	0	1
12. Heart (atrium)										
swelling of myocadium	0	0	0	0	0	0	0	1	0	1
13. Digestive tract (gastric region)										
muscularis circular necrosis	0	0	0	0	0	0	0	0	0	2
14. Digestive tract (intestine)										
cecal villi dilation	0	0	0	0	0	0	0	2	0	1
cecal villi edema	0	0	0	0	0	0	0	0	0	1
Significant level		p=0.0205			p=0.0055			n.s.		
Statistic summary[d]	f	f, e	e	h	h	g				

a Fish were ~25.6 gm in weight and held at 12±0.5°C. The volume of injection was 0.05 ml/fish.

b Fish were ~4.4 gm in weight and collected from the Oxbow Hatchery, OR by Terry D. Kreps, Fish Pathologist, Clackamas fish pathology laboratory, Clackamas, OR.

c ↓ = decreasing, ↑ = increasing

d The letters e, f, g, and h stand for a statistical summary of the groups at 4 and 7 days after injection. Values with the same letter do not have significantly different means of the number of fish with microscopic lesions. (e<f and g<h)

day 4, a blood capillary had developed and invaded the destroyed muscle. The capillary that invaded the muscle was a branch of a large blood capillary that developed in the perimysium (Figure 12). Inflammatory responses began with a leukocytic attachment to the endothelium of the invaded capillary (Figure 13). Flexibacter psychrophilus spread between myofibrils (no aggregation or massive formation) of all the infected coho salmon on days 4 and 7 (Figure 14).

The muscle tissue containing bacteria and destroyed cells was infiltrated by leukocytes. The polymorphonuclear leukocytes (PMN) were found to be the first active infiltration in the destroyed muscle tissue in the early disease condition on day 4. The phagocytic ability of PMN was limited and could not phagocytize all bacterial cells and damaged tissue. Some of the PMN cells became necrotic and had pycnotic nuclei and/or a lack of cell membrane and cytoplasm (Figure 14). The necrosis and inflammation of muscle fibers continued. The second actively infiltrating cells were mononuclear leukocytes or macrophages. Both macrophages and PMN had phagocytic activity on the bacterial cells and destroyed tissue. A proteinaceous matrix was present and was released from the myoplasm and cytoplasm of the leukocytes. A low number of bacterial cells was still present in the muscle tissue (Figure 15). The bacterial cells may have been lysed by the toxins of active macrophages, such as superoxide anion, hydroperoxyl radical, and hydrogen peroxide (Secombes, 1990).

The extensive inflammation caused a small pus formation in

Figure 10. The gill of a coho salmon infected with F. psychrophilus showing hemorrhage (H), edema (E), and hypertrophy (T). (H&E, bar=40 μm)

Figure 11. The fusion of the secondary gill lamellae of infected coho salmon caused by hyperplasia (H) of epithelial cells. (H&E, bar=40 μm)

Figure 12. Blood capillary invasion. The necrotic muscle fibers (N) have been invaded by a small capillary (S) branched from a large capillary (L) in the perimysium. (H&E, bar=40 μm)

Figure 13. High magnification of the blood capillary showed leukocytic (monocyte or macrophage) attachment (L) to the endothelium of capillary, necrotic muscle fibers (N), and edema of endomysia (E). (H&E, bar=28 μm)

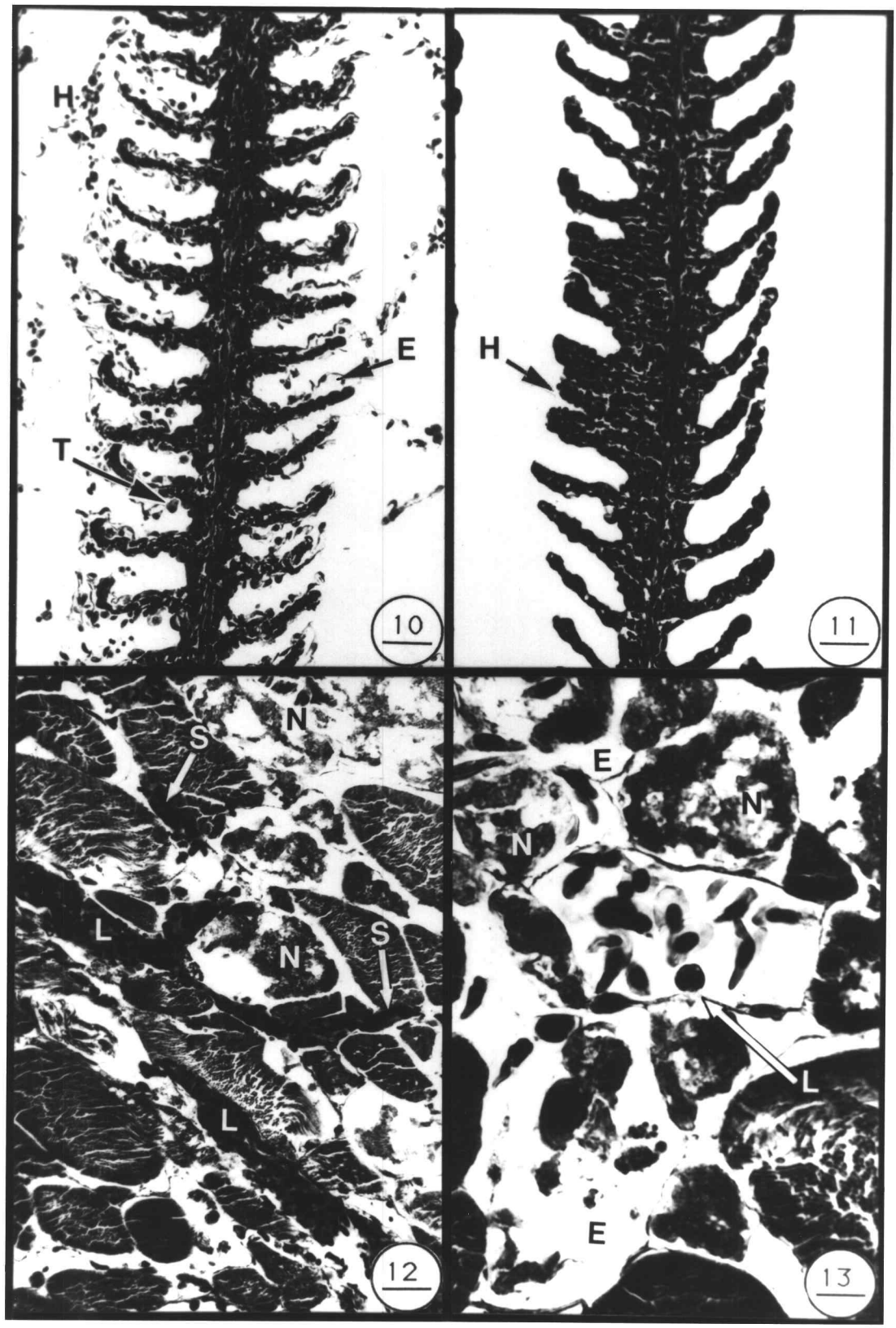

Figure 10-13

the hypodermis of the skeletal muscle and contained cell debris, proteinous substances, mixed leukocytes, and some RBC (Figure 16). This extensive lesion caused swelling of the skin. The swollen skin of infected coho salmon showed the deterioration of the epidermis, necrosis of the dermis and hypodermis, an increase of melanin, a loss of scales, and edema and atrophy of hypodermis (Figure 17). In the severe disease condition at day 7, the swollen skin around the open lesion showed an increase of fibrous connective tissue, macrophages, melanin, and RBC in the dermis with a deterioration of scale and epidermis as well as an inflammatory response of the hypodermis (Figure 18). At the crater-like or open lesion of all coho salmon infected with F. psychrophilus, the exposed hypodermis showed a severe necrosis, inflammation, hemorrhage, edema, and atrophy (Figure 19). The muscle lesions of the PBS control coho salmon also had blood capillary invasion, leukocytic infiltration, focal hemorrhage and necrosis but it was less severe and occurred in fewer fish.

Tissue, particularly muscle tissue, began to recover in the surviving coho salmon. Regeneration of the epidermis, scale, dermis, and hypodermis were found on day 19, while inflammation, necrotic tissue, and blood capillaries were absent (Figure 20). The regenerated myofibril was recognized as a small bud or small bundle, surrounded by fibrous connective tissue, and had a hyaline eosinophilic stain. The epidermis, scale, and dermis of the control coho salmon were completely regenerated by day 19, while the hypodermis was in advanced regeneration stage (Figure 21). The advanced regeneration stage consisted of a mass of fibrous

Figure 14. An inflammatory muscle fiber of infected coho salmon with infiltrated PMN (P). Some PMN are necrosis (N). The cross section view of F. psychrophilus (F) is presented. (3 μm section, Giemsa stain, bar=10 μm)

Figure 15. Progressive inflammation. The PMN (P) and macrophage (M) phagocytizied necrotic tissue and bacterial cells. Some bacterial cells (F) are still present in the muscle fibers. The proteinous (Pr) matrix increased. (3 μm section, Giemsa stain, bar=10 μm)

Figure 16. Hypodermis region of infected coho salmon skeletal muscle with marked necrosis and a small pus (S) formation which contains mixed leukocytes, some RBC, and cell debris. (H&E, bar=40 μm)

Figure 17. The swollen lesions of infected coho salmon show the deterioration of the epidermis, necrosis of the dermis and hypodermis, an increase of melanin (M), loss of scales, muscle fiber atrophy (A), and edema (E). (H&E, bar=90 μm)

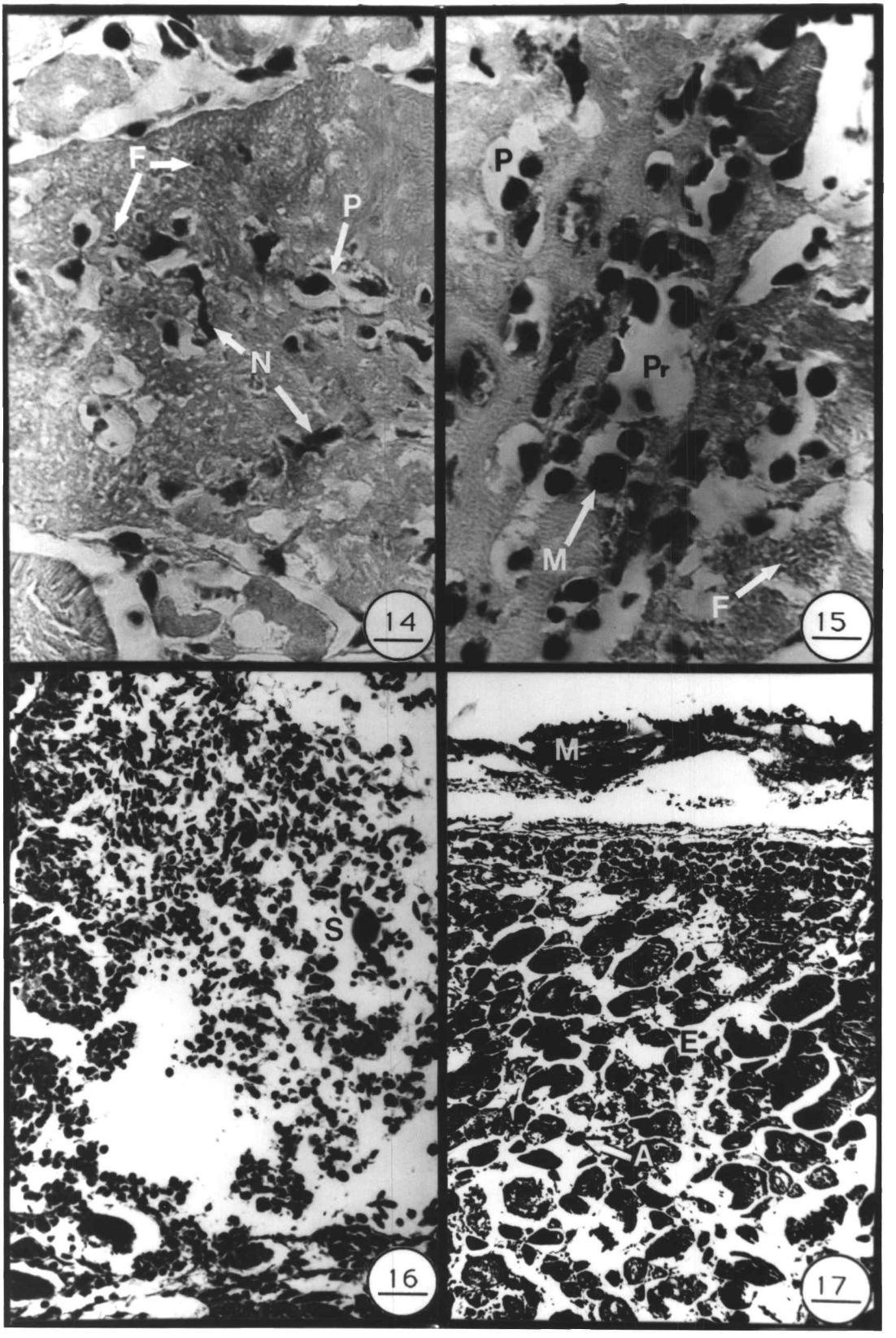

Figure 14-17

Figure 18. The high magnification of the swollen skin adjacent to the open lesion of infected coho salmon shows the deterioration of epidermis and scales, an increase of melanin (M), and hemorrhage (H) in dermis (D). The inflammation is found in both hypodermis and dermis. (H&E, bar=28 μm)

Figure 19. The open lesion of skeleton muscle of infected coho salmon shows an exposed hypodermis with severe necrosis, inflammation, atrophy, and edema. Note, a few RBC (R) were found in the lesion. (H&E, bar=40 μm)

Figure 20. The recovery of infected coho salmon at day 19 shows the disappearance of inflammed and necrotic tissue and shows the increase of fibrous connective tissue (F). The scales (S) developed and the epidermis are regenerated. (H&E, bar=123 μm)

Figure 21. The complete regeneration of the muscle of coho salmon is found in the epidermis, dermis, and scales, while the hypodermis is in an advanced regeneration stage of muscle fibers (M). The fibrous granulating tissue (F) develops between the perimysia (P). (H&E, bar=123 μm)

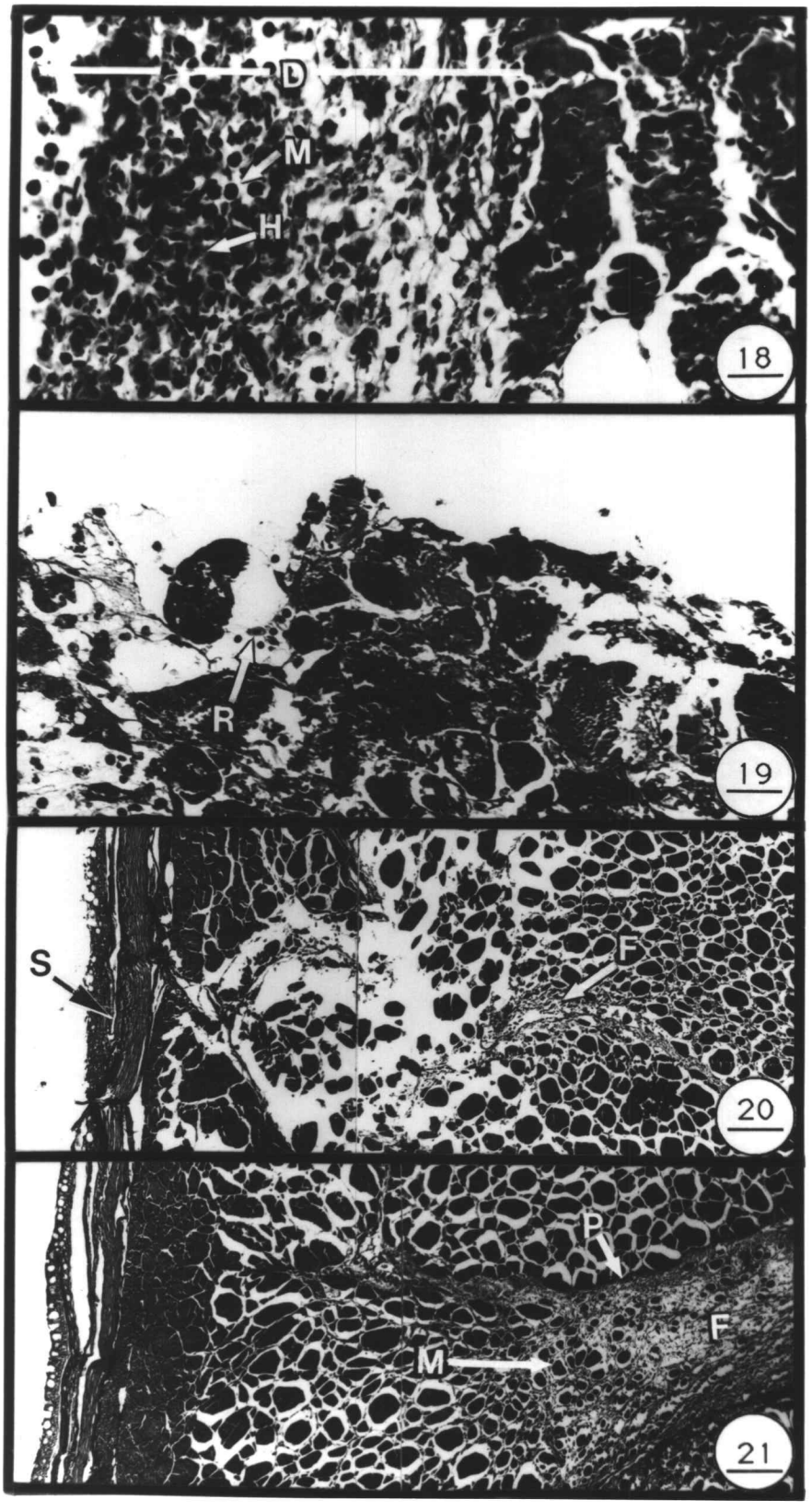

Figure 18-21

granulated tissue containing myocytes and small bundles of myofibrils located between the perimysia of muscle.

The naturally BCWD-infected coho salmon, which had swollen and opened skin, showed severe destruction similar to the experimental infection of coho salmon. The histopathologic findings consisted of blood capillary invasion, hemorrhage, leukocytic infiltration, mixed inflammation, and necrosis of muscle. A Giemsa stain section of the muscle lesions showed the long rod bacteria in the dermis and epidermis of two coho salmon. Two of five naturally infected coho salmon had atrophy of muscle fibers in the hypodermis.

The histopathologic changes of internal organs of infected coho salmon were usually found in the liver, spleen, kidney, and pancreas. The livers of coho salmon from experimental and natural infection had similar changes that included vacuolar degeneration, pycnotic nuclei, and focal hemorrhage (Figure 22). The congestion of blood sinusoids in the liver was only found at the severe disease condition and was found together with hemorrhages in some infected coho salmon at day 7 (Figure 23). Three of five of naturally BCWD-infected coho salmon developed some degree of hemorrhage in the liver, while the experimental infection was found in one fish at day 4 and the other one at day 7. The spleen of the infected coho salmon in both the bacterial injection and natural infection groups had some degree of ellipsoidal necrosis and lack of RBC in the white pulp (Figure 24).

The histopathologic changes in the kidneys of coho salmon from

experimental and natural infection included a deterioration of hemopoietic tissue in the anterior kidney and some necrosis in the renal tubules of the posterior kidney (Figure 25). Some necrosis in the stroma of reticuloendotherial tissue was found in two of five naturally infected coho salmon (Figure 26). The deterioration of hemopoietic tissue in the severe disease condition caused a decrease in the circulation of blood cells, which had been reported in the hematologic study. The accumulation of hemosiderins or melanomacrophages was slightly increased in the kidneys and spleens of infected coho salmon.

There were a few changes in the pancreases of some infected coho salmon on day 7. Necrosis of the pancreatic acinar and islets of Langerhans was found in coho salmon in the high bacterial injection group (Figure 27). A cloudy swelling of the pancreatic acinar cells was found in two coho salmon, one from the experimental infection and the other from natural infection. The following histopathologic findings from experimental infection were found in only one or two coho salmon: congestion, vacuolation, and an increase of hemosiderin or melanomacrophages in the spleen; congestion in the kidney; an increase of endocardial macrophages, swelling of the endocardium, and focal vacuolation in the cardiac muscle; and cecal villi dilation in the intestine. More coho salmon need to be examined to corroborate the effects of BCWD with these histopathologic findings. In the lower bacterial injection group, an increase of endocardial macrophages was found in two coho salmon, one from day 4 and the other from day 7, while an increase of endocardial macrophages could not be

Figure 22. The liver of infected coho salmon shows vacuolation (V) and pycnotic nuclei (P) of hepatocyte. (H&E, bar=28 μm)

Figure 23. Coho salmon liver resulting from F. psychrophilus injection shows congestion (C) of sinusoid and hemorrhage (H). (H&E, bar=28 μm)

Figure 24. Coho salmon spleen from experimental and natural infection show focal necrosis (N) of ellipsoidal cells. (H&E, bar=40 μm)

Figure 25.. The posterior kidney of infected coho salmon showss some degree of necrosis (N) in renal tubules and an increase of hemosiderin (H). (H&E, bar=28 μm)

Figure 26. The anterior kidney of infected coho salmon shows a decrease of hemopoietic tissue, an increase of hemosiderin (H), and necrosis of the stroma of reticuloendothelial tissue (S). (H&E, bar=28 μm)

Figure 27. The pancreas of infected coho salmon shows necrosis of pancreatic acinar cells (P) and islets of Langerhans (I). (H&E, bar=40 μm)

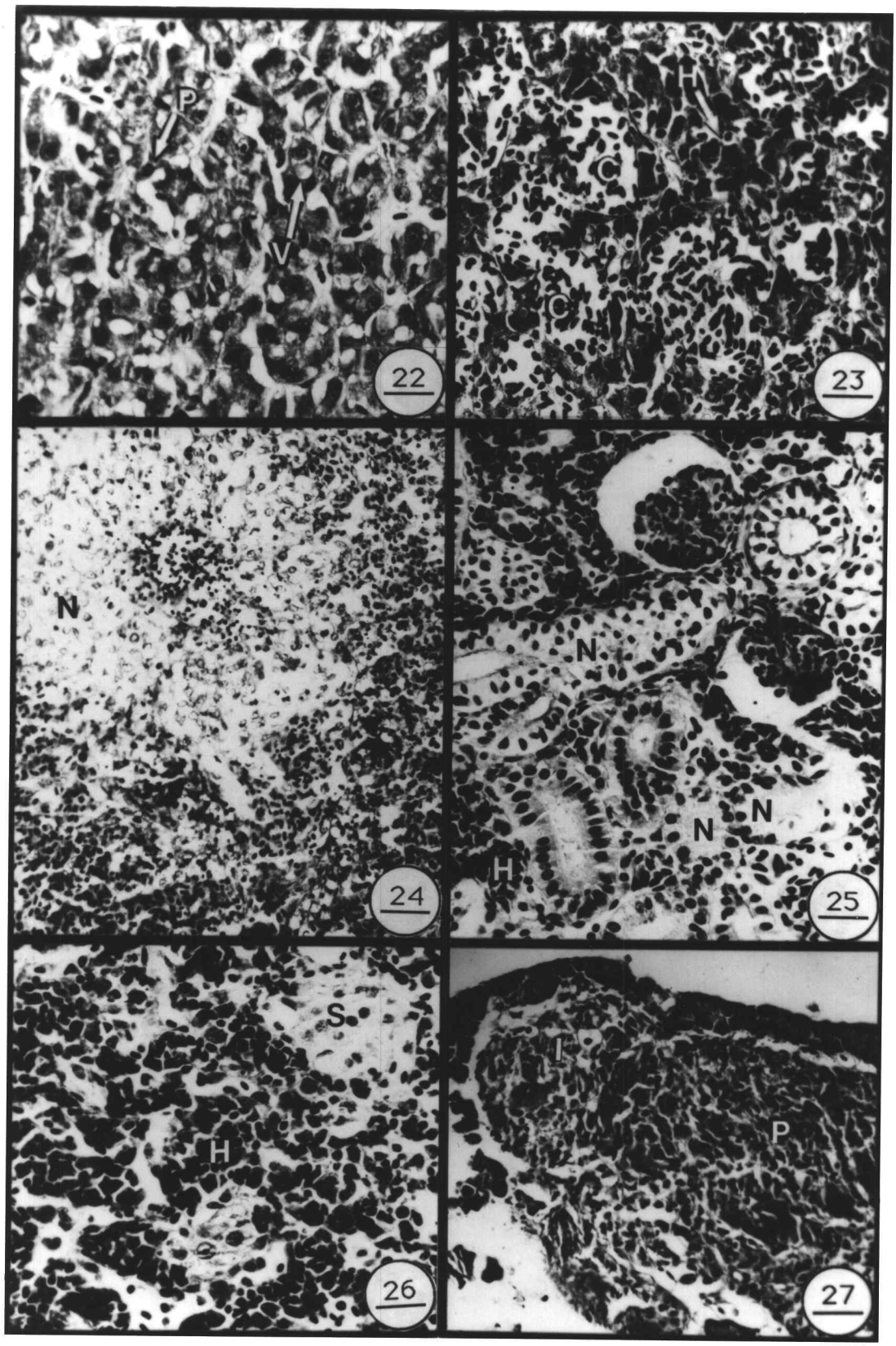

Figure 22-27

observed in the high bacterial injection group. The bacterial cells or bacteria-like particles were not found inside the endocardial macrophages. Otis (1984) reported that BCWD caused an increase in endocardial macrophages.

The other histopathologic changes in naturally infected coho salmon, which were not found in the experimental coho salmon, were hemorrhage of the visceral fat and swim bladder, necrosis of the stroma of reticuloendothelial tissue and glomeruli, thickening of nephrons, sloughing of endocardial macrophages, necrosis of the musculocircular layer of gastric region, and edema of the cecal villi of intestine.

The Giemsa-stained tissue of coho salmon from the experimentally and naturally infected groups showed the presence of the bacterium in the skeletal muscle at the site of injection. The bacterium did not colonize in the muscle tissue and could not be found in the other organs coho salmon in the severe disease condition. By using the Giemsa stain on blood smears of caudal blood vessels, F. psychrophilus was found in some infected coho salmon on days 4, 10, and 13 (Appendix 2). The number of bacterial cells in the blood smear sample was only two to eight cells per 50 microscopic fields of 1000x magnification. After the infected coho salmon recovered, the bacterium was absent from the blood stream.

DISCUSSION

Bacterial cold-water disease is a septicemic disease usually found in salmonids. The etiological agent was originally named "Cytophaga psychrophila" by Borg (1960). The classification of "C. psychrophila" and its genus has been under debate by bacterial taxonomists and presently this etiological agent is named Flexibacter psychrophilus. The definitive description of the bacterium was provided by Bernardet and Grimont (1989).

Flexibacter psychrophilus strain SH3-81 is a gram-negative slender shaped rod. Its colonial morphology on a CA plate is a fried egg-like colony. However, some strains, such as strain 144a (isolated from coho salmon in WA), form a convex colony (Holt, 1987). Motility by means of gliding can be observed by the wet mount method. An active gliding movement is dependent on incubation temperatures. The bacterium stops a gliding movement at low temperatures (12°C) and forms a tiny clump of bacterial cells in broth medium. Through comparison of six broth media, TYI and TYES gave the best growth and greatest cell mass of F. psychrophilus. Although Liewes medium had the highest optical density reading of bacterial culture, the maximum wet weight of bacterial cells was less than that obtained with TYI and TYES. This may be due to the gelatin and other compounds in the medium and/or the waste compounds or byproducts of bacterial degradation, which altered the turbidity of the culture medium. Similar growth patterns were also reported by Song et al. (1988) in another cytophagal fish pathogen, F. columnaris. They reported high optical density readings in Liewes

medium, TYI, TYE, and Cytophaga medium but did not report the maximum wet weight.

The growth of F. psychrophilus increased with increased shaking rates and incubation temperatures. The best bacterial growth was in TYI broth medium at a 130 rpm shaking rate and 18±2°C incubation temperature. Increased shaking rates may increase the dissolved oxygen in the broth medium enabling better growth of F. psychrophilus. The growth of the psychrophilic bacterium, Pseudomonas fluorescens, was faster in the aerated broth culture (Olsen and Jezeski, 1963). Although the optimal growth temperature was 15 to 20°C, the BCWD usually occurs at 3 to 15°C (Holt, 1987).

The LD_{50} of F. psychrophilus varied according to the size of the coho salmon and the length of the experiment. The LD_{50} of a 15 day experiment using coho salmon averaging 21.8 gm was 2.3×10^7 cells/ml. Holt (1987) reported that the LD_{50} of a 34 day experiment using coho salmon averaging 8 gm was 8.2×10^4 cells /ml.

Flexibacter psychrophilus is a septicemic fish pathogen, which can be isolated from the kidney and found in blood smears. The presence of F. psychrophilus in infected coho salmon was limited. Histopathologic studies showed bacterial cells were found in the kidney and muscle at the site of injection during the development of BCWD. The bacterium was also found in the blood smear. There was no colonization nor cell mass formation of this bacterium in tissue. The bacterium was rarely found after the LD_{50} experiment and could not be found on day 19 of hematologic and histopathologic experiment. Otis (1984) did not report the presence of F.

psychrophilus in any organ of steelhead trout other than the muscle at the site of injection. Bacterial rods were observed only in the muscular lesions of the naturally BCWD-infected fingerlings coho salmon from Oxbow Hatchery, Oregon, while coho salmon with the same disease at the Quilcene Hatchery, Washington had bacteria present in the pseudobranch of the gills, renal glomeruli, spleen, intestine, air bladder, peritoneal wall, and liver (Wood and Yasutake, 1956).

There is no report of hematologic study of fish infected with BCWD except for Otis (1984). He tested the extracellular products (ECPs) of F. psychrophilus strain N86 against blood cells of rainbow trout in vitro and reported that the ECPs could lyze red blood cells and reduce phagocytosis of macrophages or even lyze macrophages.

The quantitative studies of selected blood parameters of infected coho salmon revealed many changes during the severe stages of the disease. The packed cells volume was decreased. The number of RBC seemed to decrease but the statistical summary showed no changes. The reduction of RBC numbers is possibly due to hemorrhaging and deterioration of hemopoietic tissue. An increased RBC count was found during the early recovery stage when compared to fish with a severe disease condition. The decreasing of both Hct and RBC were reported from cold-water vibriosis in Atlantic salmon (Waagbø et al., 1988), natural infections by Aeromonas and Streptococcus in rainbow trout (Barham et al., 1980), bacterial kidney disease in chinook salmon (Iwama et al., 1986), and infectious hematopoietic necrosis (IHN) in rainbow trout (Amend and Smith, 1975). The hemoglobin of whole blood was decreased. The decrease

of Hb in the infected fish was also reported with several diseases of fish: rainbow trout infected with IHN virus (Amend and Smith, 1974; 1975), parasitaemia in rainbow trout (Murad and Mustafa, 1988), natural infection of Aeromonas and Streptococcus in rainbow trout (Barham et al., 1980), and bacterial kidney disease in chinook salmon (Iwama et al., 1986). The changes of Hb in BCWD coho salmon were very closely related to the changes of Hct and RBC. The same correlation was reported in brook, brown, and rainbow trout by Snieszko (1960). The decrease in these parameters showed that anemia had occurred during BCWD of juvenile coho salmon. The number of WBC or leukocytes was decreased. WBC lost from peripheral blood vessels may have moved to muscular lesions based upon the histopathological study. The decrease of WBC was also reported in the bacterial kidney disease of juvenile chinook salmon Iwama et al. (1986). They injected chinook salmon intraperitoneally with 4.1×10^7 cells/ml of Renibacterium salmoninarum and reported that the level of WBC in infected fish was significantly less than the control fish up to 12 days after the challenge. The different results were reported by Waagbø et al. (1988). They found an increase of WBC in the cold-water vibriosis of Atlantic salmon. In contrast, a viral disease, such as IHN virus in rainbow trout, caused no changes in WBC (Amend and Smith, 1975).

The plasma glucose concentration in infected fish showed differences between diseases of fish: an increase was found in rainbow trout suffering from Aeromonas and Streptococcus (Barham et al., 1980); a decrease was reported in parasitaemia of rainbow trout (Lowe-Jinde, 1979); Amend and Smith (1975) could not find

any significant differences of plasma glucose between uninfected rainbow trout and those infected with IHN virus. The quantitative analysis of glucose and total protein in the plasma of infected coho salmon showed some degree of change as the disease progressed. In the severe disease condition, glucose increased, while total protein declined. The loss of TPP is possibly due to the inflammation and hemorrhaging in the muscle lesion. The increased glucose may be due to stress from hypoxia. The severely infected BCWD coho salmon showed hypoxia with an increase rate of movement at the operculum. They had damaged gill tissue, including hypertrophy, edema, and hyperplasia. The stress effects in fish have been well documented by Mazeaud et al. (1977). The plasma glucose of infected rainbow trout with IHN virus had no changes compared with the control (Amend and Smith, 1975). Barham et al. (1980) found an decrease in TPP and glucose in rainbow trout naturally infected with Aeromonas and Streptococcus. Besides the decrease of TPP, Mulcahy (1967) reported that the electrophoretic patterns showed the lack of some specific bands of protein in a natural outbreak of ulcerative dermal necrosis in Atlantic salmon from Irish River, Ireland. Amend and Smith (1974) could not find any changes of TPP in rainbow trout infected with IHN virus

Bacterial cold-water disease causes anemia in juvenile coho salmon because of the decrease in Hct, RBC, and Hb in the severe condition. The increase of MCV showed that the anemia was caused by the lack of young RBC in the peripheral blood circulation and the loss of RBC at the hemorrhagic muscle (as shown later in the histopathologic study). Harding and Høglund (1983) reported that

the young RBC was smaller than the mature RBC in the blood circulatory system. The decreased MCHC may be due to the decrease of hemoglobin synthesis. The histopathologic examination found the decrease of hemopoietic tissue in kidneys that caused the deficiency of RBC production and hemoglobin synthesis. From these characteristics, the anemia in BCWD coho salmon should be classified as hypoplastic anemia according to Ferguson (1990) and Roberts (1978). The changes of MCV, MCH, and MCHC differs in different diseases of fish: natural infection of rainbow trout with Aeromonas and Streptococcus caused decreased MCH and no changes in MCV and MCHC (Barham et al., 1980); cold-water vibriosis of Atlantic salmon caused decreased MCV and no changes in MCH and MCHC (Waagbø et al., 1988); metacercaria in male catfish caused decreasing MCH and MCHC (Murad and Mustafa, 1988); infectious hematopoietic necrosis virus disease in rainbow trout caused no changes in any of the three parameters (Amend and Smith, 1975).

The differential count of WBC in BCWD coho salmon showed some change during the progression of the disease. In the early disease condition, the blood monocytes decreased in percent and number from 6.0 to 1.0% and 580 to 80 cells/mm^3, respectively. The decreased number of monocytes in the bloodstream may have been transformed to macrophages, and accumulated in the damaged tissue of the muscle and performed active phagocytosis. Monocytes have been recognized as the precursor of macrophages by Ellis and Roberts (1978). The increased number of macrophages in muscle tissue was clearly shown in the histologic study and counted as a secondary leukocytic invader. Although the PMN or neutrophils

were the primary leukocytic invader, their accumulation in muscle tissue was not large enough to reduce their cells in peripheral blood circulation, while the monocyte accumulation was. On the other hand, the rate of PMN production may equal the rate of PMN emigration. The percent of peripheral thrombocytes increased from 37.0 to 51.2% in the severe disease condition in order to heal the wound and stop the bleeding in the damaged muscle tissue. Thrombocytes also have some phagocytic ability (Ferguson, 1976). In the early stage of recovery, the number of lymphocytes and neutrophils increased from 4.5 x 10^3 to 9.0 x 10^3 cells/mm^3 and 0.4 x 10^3 to 1.3 x 10^3 cells/mm^3, respectively. The percent of lymphocytes also increased from 42 to 70% on day 13. The increase of lymphocytes and neutrophils suggested that they play an important role in eliminating the remaining F. psychrophilus in coho salmon during the recovery stage of the BCWD. The viral disease, IHN, of rainbow trout caused increased numbers of lymphocytes, decreased numbers of neutrophils, and no changes in monocytes (Amend and Smith, 1975). The parasitic disease, metacercaria of Diplostomulum sp., in catfish caused increased numbers of neutrophils, monocytes, eosinophils, and doubly increased numbers of lymphocytes (Murad and Mustafa, 1988).

The values of the hematologic parameters fluctuated during the different stages of the BCWD conditions. This hematologic study suggested that the report of hematologic changes in diseases of fish should specify the stage of the disease condition. Future hematologic studies should involve the qualitative changes of the plasma protein.

The gross lesions of infected coho salmon were mainly observed at the site of injection beginning with the black patchy color of melanin, swollen skin, and ending with open lesions. The internal organs (liver, kidney, and spleen) had some changes related to hemorrhaging. Coho salmon naturally infected with BCWD had less severe muscle lesions but more severe changes in the internal organs. These gross lesion findings were similar to the findings of Otis (1984). Both studies found hemorrhage as a major change in every clinical organ. In Otis' (1984) experiment he also found a petechial hemorrhage of the peritoneal wall and hemorrhage of the swim bladder. Schachte (1983) reported similar gross lesions in fingerling lake trout naturally infected with BCWD. The lesions began with a black patchy area at the caudal peduncle, erosion of the caudal fin, opening and sloughing of skin at the caudal peduncle and sometimes ending with disappearance of the caudal fin.

The histopathologic lesions of infected coho salmon showed many changes related to the developmental stages of the disease. Both high and low dose injections (9.4×10^5 and 2.5×10^7 cells/ml, respectively) caused severe lesions at about the same time, on day 7. The severe lesions appeared from day 7 to 10 in the higher dose injection.

In the early disease condition on day 4, the muscle fibers had necrosis, blood capillary invasion, and inflammation at the site of the injection, resulting from the bacterial injection. The invaded blood capillary was adjacent to the necrotic muscle tissue. Capillary invasion in the surgical wound experiment of Atlantic salmon had been reported as early as 31 hours and disappeared after 18 days

(Anderson and Roberts, 1975). The leukocytes emigrated across the wall of the blood capillary and infiltrated into the necrotic tissue. The histopathologic study muscle tissue suggested that the PMN is the first leukocytic infiltration and the macrophage or mononuclear leukocyte is the second. The increased number of macrophages was a result of the transformation of the monocytes in the peripheral blood vessels (no attempt was made to determine the percentage of the monocytes). Finn and Nielsen (1971b) reported that the infiltration of PMN occurred as early as 12 hours after intramuscular injection of rainbow trout with heat-killed Staphylococcus aureus, while the infiltration of macrophages was found after 24 hours. The inflammatory responses and rate of healing increased with an increased incubation temperature of the holding water (Finn and Nielsen, 1971a).

The PMN and macrophage infiltrations were also found in the naturally infected coho salmon and in the control group. Infiltration in the latter group was less severe. The bacterial cells and necrotic tissue were removed by the phagocytosis of PMN and macrophage. A severe inflammation had a small amount of pus which contained cells debris, proteinous substances, mixed leukocytes, and RBC. The lymphocytes and thrombocytes are lacking in leukocytic infiltration and phagocytosis ability. This was also reported by Nielsen (1971b). Pus was released after the lesion had been opened. Holt (1987) used a scanning electron microscope to examine the open lesions of naturally BCWD-infected in coho salmon. He found disorganized muscle fibers and spreading bacterial cells.

In the recovery disease condition at day 19, myofibril regeneration was found in the fibrous granulating tissue between the perimysia. In the wound healing experiment of Anderson and Roberts (1975), myofibril regeneration occurred after 18 days. In the inflammatory response experiment of Finn and Nielsen (1971b), myofibril regeneration was found at day 16.

The coho salmon experimentally and naturally infected had necrosis of muscle tissue, hypertrophy and edema of the gills, and some necrosis of the renal tubules. These histopathologic lesions suggested that the infected coho salmon sustain muscle lesions and a decrease in oxygen uptake and ion-exchange of the gills. At this stage of the disease, the infected coho salmon showed hypoxia, an increased rate of operculum movement. The bacterial cells could not be observed in the gills and renal tubules which suggested that bacterial products or toxins are involved. Otis (1984) reported that the extracellular products of F. psychrophilus played a major role in the histopathologic lesions of BCWD in steelhead trout. Although the hemorrhaged tissues are usually found in the organs of infected coho salmon, the hemorrhaged areas are small. The soft and bloody tissue in the swollen and opened skin did not show a severe hemorrhage when examined histologically. The coho salmon infected with Flexibacter psychrophilus did not have a severe hemolysis of RBC because there was a little increase of hemosiderin or melanomacrophages, which accumulated in the spleen and kidney.

The decreased amount of hemopoietic tissue in the anterior kidney of infected coho salmon was the major cause of hypoplastic anemia. The histopathologic change of the kidney may be due to an

accumulation of bacterial toxins. According to Ferguson et al. (1982), the kidney is a major site of bacterial clearance from the bloodstream. Agius (1979) reported that the melanomacrophage center in the spleen had a large amount of iron while it was almost absent in the kidney. Roberts (1978) reported that this kind of anemia could be found in the fish with IHN virus infection, tuberculosis, bacterial kidney disease, proliferative kidney disease, and prolonged treatment with chloramphenicol and sulphonamides.

The pycnotic nuclei of hepatocytes are usually observed in both experimentally and naturally BCWD-infected coho salmon. This stage of necrosis is common in septicemic and toxic diseases (Ferguson, 1990). The vacuolar or hydropic degeneration of hepatocytes in all experimental groups may be caused by PBS. Flexibacter psychrophilus seems to cause the additive effect of vacuolar degeneration. Because PBS was injected in the control groups and used as a diluting solution of bacterial injection groups, this degeneration may be caused by the failure of the sodium pump that changed the electrolytic charge of protein in the cytoplasm (Roberts, 1978). Flexibacter psychrophilus can cause blood congestion in the sinusoids and hemorrhaging as reported by Otis (1984). He found that four of five steelhead trout infected with BCWD showed congestion and dilation of liver. Bacterial cold-water disease caused splenic ellipsoid necrosis in both experimental and natural coho salmon. One function of the spleen is as a purification site of foreign material in the bloodstream by phagocytic activity of macrophages in the ellipsoid (Ellis and Roberts, 1978), another is blood storage. The ellipsoid necrosis may limit the ability of blood

infiltration and purification. About two-fifths of the blood circulation, which increased in the exercised fish, came from the spleen (Yamamoto et al., 1979). Ferguson (1990) reported this histopathologic change in furunculosis of brown trout.

The infected coho salmon lost their appetite in the severe disease condition, which may due to the decrease or lack of pancreatic enzyme production. Flexibacter psychrophilus caused necrosis and atrophy of the pancreatic acinar, which may reduce the pancreatic enzyme producing granules or zymogens granules. The bacterium also caused necrosis in the islets of Langerhans, the insulin producing tissue. According to the hematologic study, the increased plasma glucose in the severely infected coho salmon may be due to a lack of insulin secretion from the islets of Langerhans. Yasutake and Wales (1983) reported that the secretion of insulin in trout could be inhibited by the injection of alloxan (an oxidized product of uric acid which causes a necrosis of the islets of Langerhans), which causes an increase of plasma glucose. Further studies should examine the relationship between insulin and plasma glucose of the fish infected with BCWD or another disease, such as IPN virus disease.

SUMMARY AND CONCLUSIONS

Morphology: Flexibacter psychrophilus strain SH3-81 is a slender, rod shaped, gram-negative bacterium, about 0.3 to 0.5 μm in width and 2 to 7 μm in length and exhibits a fried egg-like colony, glossy convex at the center with a thin spreading margin, on Cytophaga agar after 4-5 days incubation at 15±1°C.

Culture characteristics: Tryptone yeast infusion (TYI) and tryptone yeast extract (TYE) plus salts give good growth. The bacterium grows better at 18±2°C than 15±1°C incubation temperature. The bacterium grows better at 130 than 100 and 70 rpm shaking rates.

LD_{50}: The medium lethal dose (with a 95% confidence interval) of this bacterium is 2.3×10^7 (8.5×10^6 to 6.2×10^7) cells /ml injected intramuscularly in coho salmon averaging 21.8±4.0 gm at day 15 and 12±0.5°C incubation temperatures.

Hematology: The values of hematologic parameters of the infected coho salmon changed during the progression of the disease. The following are the values of the parameters at the severe disease condition of coho salmon that have significantly different means

compared with the control group (the control values are in the parentheses); hematocrit is 31.0 (39.9%); red blood cell count is 1.06 x 10^6 (1.51 x 10^6 cells/mm^3); white blood cell count is 2.6 x 10^3 (9.7 x 10^3 cells/mm^3); total plasma protein is 1.25 (4.00 gm/dl); hemoglobin is 8.23 (9.96 gm/dl). The plasma glucose concentration is highest in the severe disease condition (139.3 mg/dl) then levels off at recovery (86.5 mg/dl). The mean corpuscular volume significantly increased to 368 μm^3/cell at the severe disease compared with 278 μm^3/cell of in the early disease condition and 264 μm^3/cell of the recovery. The mean corpuscular hemoglobin significantly increased to 83 pg/cell compared with 68 pg/cell of the control group. The mean corpuscular hemoglobin concentration of the severely infected coho salmon is 23.4% which is significantly lower than that of the recovered coho salmon (28.6%).

In the early disease condition, the percent and absolute count of monocytes significantly decreased from 6.0 to 1.0% and 580 to 80 cells/mm^3 (P=0.0177 and P=0.0124), respectively. The percent of thrombocytes significantly increased from 37.0 to 51.2% (P=0.0358) in the severe disease condition. In the early stage of recovery, the absolute count of lymphocytes and neutrophils significantly increased from 4.5 x 10^3 to 9.0 x 10^3 and 0.4 x 10^3 to 1.3 x 10^3 cells/mm^3 (P=0.0453 and P=0.0453), respectively. These increased values returned to the same levels as the control by day 13. The percent of lymphocytes significantly increased from 42.0 to 70.0% (P=0.0360), in the recovery stage of the disease. The decreased amount of monocytes in the early stage of the disease is believed to be localized in the muscle at the site of bacterial injection. The

lymphocytes and neutrophils played an important role in phagocytosis and/or immune response to the disease in the recovery stage.

Histopathology: The lesions of infected coho salmon progressively developed at the site of injection beginning with a black patch of melanin, swollen skin, and ending with an open lesion of the skin. An inflammatory response occurred after injection. The large blood capillary invasion in the perimysium developed before 4 days after injection and inserted a small capillary between the endomysia of the destructive muscle fibers. The destructive muscle fibers were infiltrated by polymorphonuclear and mononuclear leukocyte. Flexibacter psychrophilus spread between the myofibrils. The bacterium can cause swollen lesions by day 4 with deterioration of the epidermis and scales, necrosis of the dermis and hypodermis, an increase of melanin, atrophy of muscle fiber, edema, and pus formation. The open lesion was found on day 7 with severe necrosis, inflammation, atrophy, edema, exposed hypodermis, and a low degree of hemorrhaging. The early myofibril regeneration of infected coho salmon was found on day 19, while the control group was in the progressive stage. The bacterium caused histologic changes of the following organs: the gills with hypertrophy, edema, and hemorrhage; the liver with vacuolar degeneration, pycnotic nuclei, congestion, and hemorrhage; the spleen with ellipsoidal necrosis, congestion, increasing hemosiderins or melanomacrophages, and vacuolar degeneration; the kidney with decreasing hemopoietic tissue, increasing hemosiderins, renal tubules necrosis, and

congestion; the pancreas with necrosis of the acinar and islets cells. The infected coho salmon have low incidence of focal vacuolation of the cardiac muscle, increasing and swelling of endocardial macrophages, and cecal villi dilation.

The naturally BCWD-infected coho salmon have similar histopathologic lesions but they are less severe in the muscle lesions and more severe in the internal organ lesions. The lesions, which could not be found in the experimental coho salmon, are hemorrhaging of the visceral fat and swim bladder, necrosis of the stroma of reticuloendothelial tissue, thickening of the renal tubules, necrosis of the muscularis circular of gastric region, and edema of the cecal villi.

Flexibacter psychrophilus caused hypoplastic anemia in coho salmon. The bacterial cells were eliminated from the body of the surviving coho salmon in the early stage of recovery on days 10 and 13 of the lower and higher bacterial infection groups, respectively.

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APPENDICES

Appendix 1. The procedures of LD_{50} calculation according to Litchfield and Wilcoxon (1949) with minor modifications.

Dose (cells/ml)	Died/tested	Observed% mortality	Expected% mortality	Observed minus Expected	Contribution to X^2 from nomograph 1 (not shown)
5.3×10^8	18/20	90	88	2	0.0035
2.1×10^8	15/20	75	77	2	0.0022
5.8×10^7	11/20	55	62	7	0.0200
6.6×10^5	7/20	35	35	0	0
9.5×10^5	2/20	10	9.5	0.5	0

Total coho salmon = 100 Total = 0.0257

Number of dose, k = 5 $X^2 = 0.0257 \times 20 = 0.514$

Total coho salmon/dose = 100/5 = 20 Degree of freedom (D.F.) = k-2 = 3

The total X^2 is 0.514 which less than 7.82 of X^2 value at p=0.05 and D.F.=3; therefore, the estimate line in Figure 9 is good enough to represent the accumulation mortality.

$LD_{84} = 3.9 \times 10^8$ cells/ml

$LD_{50} = 2.3 \times 10^7$ cellc/ml

$LD_{16} = 1.5 \times 10^6$ cells/ml

Slope function (S) = $\frac{LD84/LD50 + LD50/LD16}{2} = 16.15$

N' = The number of coho salmon used between 16 and 84% expected effects = 60

Facter LD_{50} (fLD_{50}) = $S^{2.77/\sqrt{N'}} = 16.15^{2.77/\sqrt{60}} = 2.70$

A 95% confidence interval of LD_{50}

Upper limit = $LD_{50} \times fLD_{50} = 6.2 \times 10^7$ cells/ml

Lower limit = $LD_{50} / fLD_{50} = 8.5 \times 10^6$ cells/ml

The LD_{50} and 95% confidence interval of F. psychrophilus strain SH3-81 in coho salmon averaging 21.8±4.0 gm are 2.3×10^7 (8.5×10^6 to 6.2×10^7) cells/ml 15 days after intramuscular injection (12±0.5°C incubation temperatures).

Appendix 2. The number of coho salmon[a] with bacteria present in the blood smear[b] after intramuscular injection with 9.4×10^5 and 2.5×10^7 cells/ml of Flexibacter psychrophilus cells.

Day(s) after injection	Viable cells injected (cells/ml)		
	PBS Control	9.4×10^5	2.5×10^7
1	NE[c]	0	0
4	NE	2[d]	2
7	NE	3	4
10	NE	0	4
13	NE	0	0
19	NE	0	0

a Fish were ~25.6 gm in weight and held at 12±0.5°C. The volume of injection was 0.05 ml/fish.

b Giemsa stain for blood smear.

c NE=not examined

d The bacterial cells were found between 2 to 7 cells in the area of 50 microscropic fields of 1000x magnification.